ADVANCES IN VIRUS RESEARCH, VOL. 32

ADENO-ASSOCIATED VIRUSES: AN UPDATE

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I. Introduction

The adeno-associated viruses (AAV) are the only known DNA animal viruses which are absolutely dependent upon coinfection by a second unrelated virus in order to undergo productive infection. They are members of the family Parvoviridae, which are among the smallest of the DNA animal viruses (Siegl *et al.*, 1985). The Parvoviridae genome is a linear single-stranded DNA molecule approximately 5 kb in size which is encapsidated in a naked icosahedral particle 18–27 nm in diameter. In addition to one genus of insect viruses (densoviruses) the family contains two genera that infect a broad spectrum of vertebrates ranging from birds to man. The parvoviruses are able to replicate autonomously in infected cells but require actively dividing cells for a productive infection. Although the dependoviruses (AAV) are structurally similar to the autonomous parvoviruses, they are absolutely defective and require coinfection with structurally unrelated adenoviruses or herpesviruses for a productive infection to occur (Atchison *et al.*, 1965; Melnick *et al.*, 1965; Hoggan *et al.*, 1966; Buller *et al.*, 1981). Adeno-associated virus does not have any structural relatedness to either of its helpers; on the other hand, the three viruses do represent all of the known vertebrate virus families with linear DNA genomes that replicate in cell nuclei.

Several significant questions arise with regard to the biology of AAV. (1) AAV is structurally unrelated to either of its helpers. With the exception of rather short sequences (albeit in rather interesting regulatory regions) there is an absence of significant homology among the DNAs of the three viruses (Rayfield *et al.*, 1986). Yet the requirement of helper virus coinfection indicates a function or functions possibly shared in common. In fact, all aspects of AAV macromolecular synthesis are affected by the helpers. Thus, is there a rather striking universality in spite of the apparent differences? (2) AAV is widespread in the human population and in other species, yet has never

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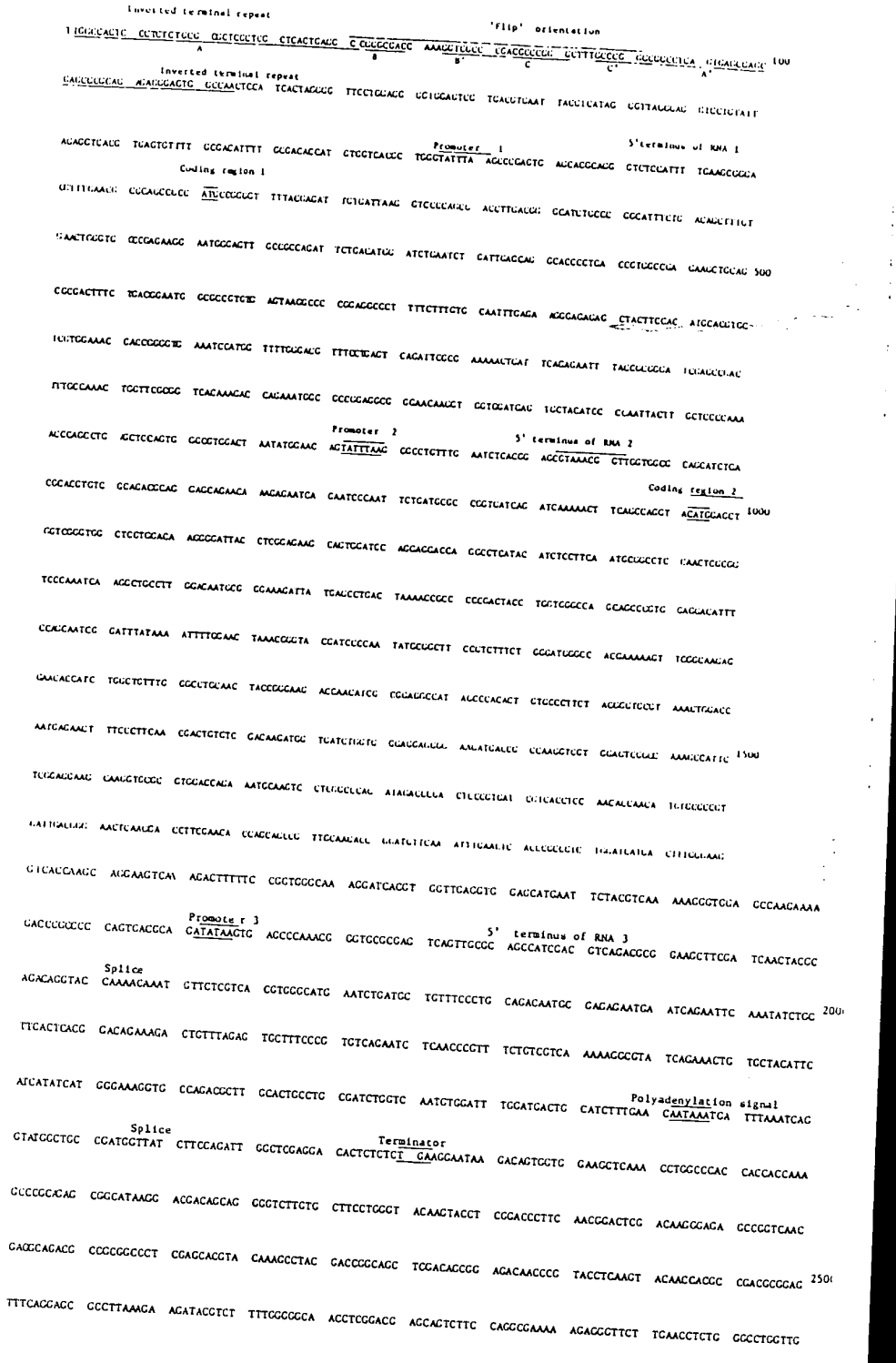
been associated with any disease. What are the potential consequences for the host of AAV infection? Tied in with this is the question of the balance between AAV and its helper viruses, the consequences of the presence of AAV, as well as the normal host responses for either an adenovirus or a herpesvirus infection. (3) Because it is defective, AAV faces a special problem in terms of the biological continuity of its genome—the helper virus will not always be present. How the AAV replication cycle is capable of overcoming this potential problem is of major interest.

The aim of this article is to present our current knowledge about the basic mechanisms underlying AAV replication in light of the questions posed above with a particular emphasis on the explosive increase in information that has been made possible in the last several years by the advent of recombinant DNA technology.

II. Background

Adeno-associated virus was discovered in the late 1950s as a contaminant of adenovirus preparations by observation in the electron microscope. Although initially considered to be either a precursor or a breakdown product of the adenovirus virion, by the mid-1960s AAV was determined to be a distinct virus which was dependent on adenovirus coinfection for its own multiplication (Atchison *et al.*, 1965; Melnick *et al.*, 1965; Hoggan *et al.*, 1966). Subsequently it was discovered that various herpesviruses could also function as helpers, although the question of whether infectious viruses were being produced was unclear. More recently it has been reported that at least Herpes simplex viruses (HSV) I and II can function as total helpers (Buller *et al.*, 1981). To date, though, AAV has only been isolated from people and animals undergoing a concurrent adenovirus infection (Blacklow *et al.*, 1968a,b). Thus, the frequency with which herpesviruses may serve as helpers in the natural state is unclear. Adeno-associated virus has been isolated from a number of species and the thought is that AAV may potentially be isolated from any species for which there is an infectious adenovirus. Again, whether AAV is present in species which are infected by herpesviruses but not adenoviruses (if any such exist) also is unknown. The situation is somewhat complicated by the fact that in cell culture AAV is not specific for its normal host species; e.g., human AAV may be replicated in canine cells coinfected with a canine adenovirus (Hoggan *et al.*, 1966). Although AAV does not appear to be completely species nonspecific, this type of broad host range supports the notion of a commonality of a variety of functions critical

FIG. 1. Nucleotide sequence of adeno-associated virus type 2 DNA. Bases 3758–3763 were not present in the original sequence but were discovered upon resequencing by R. Wright and N. Muzyczka (personal communication). (Reprinted with permission from Srivastava *et al.*, 1983, *J. Virol.* **45**, 555–564.)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A
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B'
C
C'
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Inverted terminal repeat
Promoter 1
5' terminus of RNA 1
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5' terminus of RNA 2
Coding region 2
Promoter 3
5' terminus of RNA 3
Splice
Splice
Polyadenylation signal
Terminator

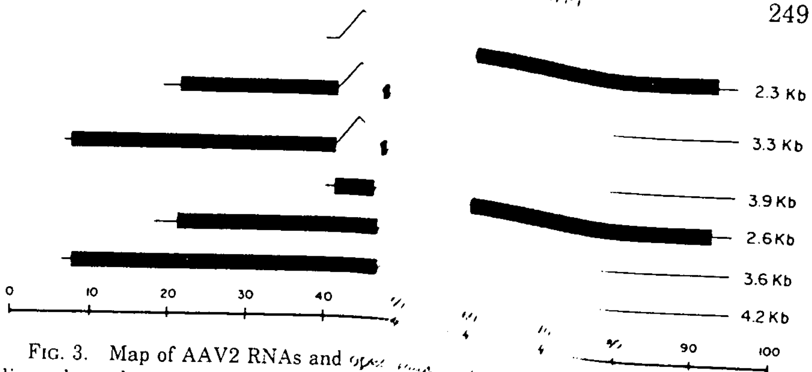

FIG. 3. Map of AAV2 RNAs and [illegible] lines above the genome represent AAV [illegible] genome. The solid and unspliced (2.6., 3.6, and 4.2 kb) form [illegible] 2.3. 3.3., and 3.9 kb) within these RNAs. (Reprinted with pe[illegible] open reading frames 329–339.) [illegible] 1984, *J. Virol.* 51,

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(Rose *et al.*, 1969; Mayor *et al.*, 1969; Berns and Rose, 1970; Berns and Adler, 1972). Upon extraction, the complementary strands can base pair to form duplex DNA. This phenomenon has been of assistance in various studies of DNA structure. In contrast to the autonomous parvovirus genome, the AAV genome has an inverted terminal repeat of 145 bases (Kozcot *et al.*, 1973; Gerry *et al.*, 1973; Berns and Kelly, 1974; Lusby *et al.*, 1980). The terminal 125 bases form an overall palindrome which is interrupted from bases 41–84 by two small 21-base palindromes. As a consequence, when this terminal 125-base sequence is folded on itself so that maximum base pairing can occur, the T-shaped structure illustrated in Fig. 2 is formed. Of particular note in this structure are that (1) only seven bases are unpaired, six of which are needed for the hairpinning required to form the crossarms of the T-shaped structure, and the seventh serves to separate the two crossarms; and (2) all of the base pairs within the crossarms, with a single exception, are GC pairs. The sequence of the terminal repeats is also heterogeneous; only 35% of the 5′ termini have the sequence TTG; 50%

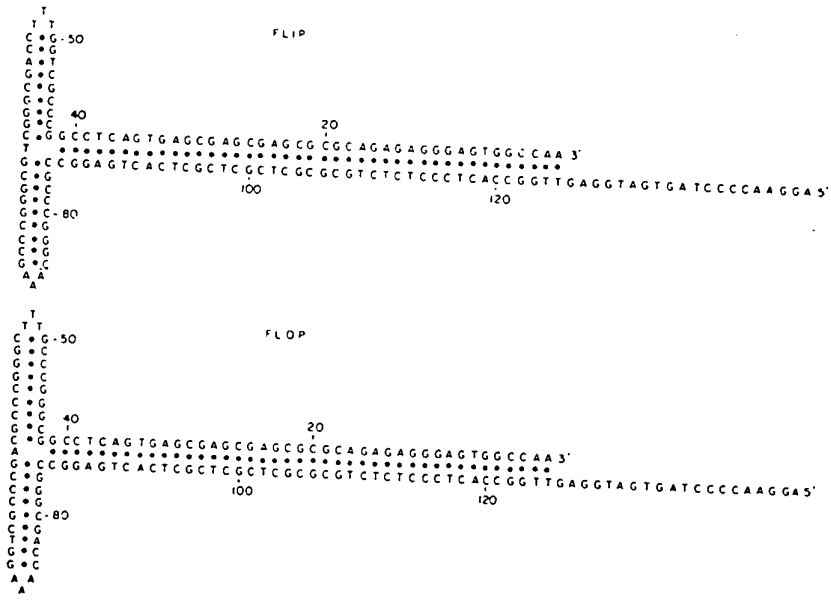

FIG. 2. Structure of the 145 nucleotides of the AAV type 2 terminal repeat in its maximally based paired configuration. Two possible sequence orientations exist and they are designated "Flip" and "Flop." (Reprinted with permission from Lusby *et al.*, 1980, *J. Virol.* 34, 402–409.)

A. Helper Virus Functions

Because of the apparent differences between AAV and its helpers and the differences between the helpers themselves, the simplest notion would be that AAV replication requires a single helper function. This idea would also be in accord with the fact that the autonomous parvoviruses indeed appear very similar in structure to AAV. However, we now know that the actual case is far more complex. Indeed all phases of AAV-specific macromolecular synthesis, including RNA transcription, DNA replication, and protein synthesis, require helper virus functions. Because of its relative simplicity, the genetics of the adenovirus genome are much better understood than are those of Herpes simplex virus. Thus, studies with regard to specific helper functions provided for AAV replication are much better understood in the case of adenovirus. Adenovirus *E1a* gene product is required for AAV transcription (Laughlin *et al.*, 1982; Tratschin *et al.*, 1984b). The adenovirus *E1b* gene has a less certain role in AAV replication. One mutant has been reported to support AAV DNA replication (and hence transcription) but not to rescue AAV from the integrated state in latently infected cells (Ostrove and Berns, 1980). This mutant is DNA negative for adenovirus (Harrison *et al.*, 1977). Other mutants in *E1b* have been reported not to support AAV DNA replication (Laughlin *et al.*, 1982). Early region 4 of adenovirus is required for AAV DNA replication but not for adenovirus DNA replication (Janik *et al.*, 1981; Richardson and Westphal, 1981). This region codes for several polypeptides. It appears that the 25-kDa protein is the critical one for AAV. Adenovirus early region *2a*, on the other hand, codes for a single-stranded DNA binding protein required for adenovirus DNA synthesis but not for AAV DNA synthesis. However, mutants in this gene affect adenovirus host range at the level of synthesis of some structural proteins (Klessig, 1977; Klessig and Grodzicker, 1979; Kruijer *et al.*, 1981) and have been reported to affect production of infectious AAV virions, although this observation has been a source of disagreement among different laboratories (Handa *et al.*, 1975; Straus *et al.*, 1976a; Myers *et al.*, 1980; Myers and Carter, 1981; Jay *et al.*, 1981; McPherson *et al.*, 1982; Carter and Laughlin, 1984). Adenovirus codes for two small RNA polymerase III-transcribed virus-associated (VA) RNAs which are required for optimal protein synthesis by both adenovirus (Thimmappaya *et al.*, 1982) and AAV (Janik *et al.*, 1981, 1982). Thus, in spite of some disagreement about details, it is clear that adenovirus does provide a wide variety of helper functions for AAV replication which affect every level of the process. The process is much

ORFs and the three transcripts are finally expressed as at least five proteins, a striking example of the economy of function within the small AAV genome.

B. Proteins

There are three AAV coat proteins: VP1, 87 kDa; VP2, 73 kDa; and VP3, 62 kDa (Johnson *et al.*, 1971, 1975; Rose *et al.*, 1971; Salo and Mayor, 1977). The amino acid sequences of the three proteins overlap extensively; essentially all of the sequences of the smaller species are contained within the sequences of the larger species (Rose *et al.*, 1971; Johnson *et al.*, 1977, 1978). The coat proteins are neither phosphorylated nor glycosylated, although there is evidence for acetylation. Determination of the amino acid sequences of any of these proteins has been confounded to date by their insolubility in aqueous solvents and by the fact that the 5′ termini are blocked. The manner in which it is currently thought that these three proteins may be generated from a single ORF is detailed below in Sections IV,B and IV,E on replication and genetics, respectively. The situation is further complicated by reports that multiple species of VP1 and VP3 can be resolved on SDS–polyacrylamide gels (McPherson and Rose, 1983). Whether this heterogeneity in mobility reflects small differences in molecular weight or different extents of posttranslational modification(s) is unknown.

IV. Replication

On the one hand, the small size and simple structure of the AAV virion serve to enhance studies of virus replication. On the other hand, there are two factors which tend to complicate the situation. The first is that the very small size of the genome leads to a situation in which many of the essential functions for replication overlap, and thus dissection is rendered more difficult; removal or mutation of any sequence is liable to affect more than one function. The second is that the requirement for a helper virus means that an additional set of functions must be identified and characterized. The situation is rendered more complex by the fact that the relationship between AAV and its helpers is a dynamic one so that not only is AAV replication affected by the helper, but the reverse is also true: replication of the helper is affected in many instances by the concomitant replication of AAV.

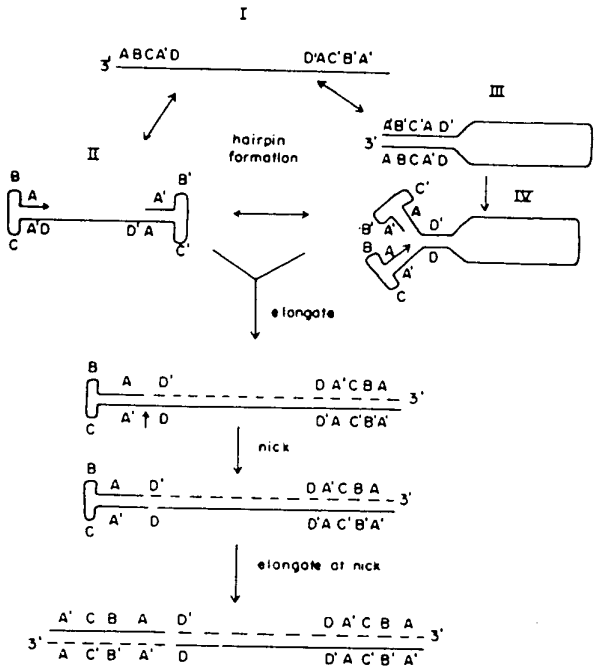

FIG. 4. Model for AAV DNA replication. See text for details.

Because of the inverted repeat, the same sequence is at the 3′ ends of both DNA strands so that the primer is in common. (3) Features 1 and 2 confer an extensive capacity for self-repair on the ends. Within the first 25 bases, therefore, there are the equivalent of four copies of one sequence. Overall there are two copies, by definition, of the inverted terminal repeat. As an example, pSM609 is a recombinant plasmid in which 113 bases have been deleted from the left end of the AAV genome and 9 bases from the right end (Fig. 5). Yet this plasmid is viable, infectious progeny are produced, and the progeny genomes have the wild-type sequence at both ends (Samulski *et al.*, 1983). Several models are possible to explain this result. One is as follows: The right can still hairpin and serve as a primer. The first 9 bases inserted effectively repair the deletion on the right end. The 32 bases that remain of the inverted repeat will be at the 3′ end of the progeny strand and able to base pair with the intact inverted terminal repeat at the 5′ end of the progeny strand. The 5′ overhang resulting can then be used as a template for extension and repair of the 3′ end. Thus, as long as a potential template remains, a deletion within the terminal repeat

less defined in the case of required HSV functions. Mutants have been identified which do not support AAV DNA replication, and preliminary studies have shown that one or more of the HSV immediate early genes can turn on AAV transcription (Tilley and Mayor, 1984; M. A. Labow, S. Silverstein, and K. I. Berns, unpublished observations).

B. Genetics

Classical approaches to the genetics of AAV are exceedingly difficult, if not impossible, in a practical sense because of the defectiveness of the virus. Fortunately, the techniques of recombinant DNA technology and the biology of the virus now render genetic analysis of the AAV genome a relatively straightforward process, probably one of the easiest for any animal DNA virus. This is the consequence of the fact that when the intact double-stranded form of the AAV genome is cloned into a bacterial plasmid such as pBR322, the resulting clone is biologically active when transfected into human cells in culture (Samulski *et al.*, 1982; Laughlin *et al.*, 1983). If the cells are coinfected with helper adenovirus the AAV genome is rescued from the recombinant plasmid, replicated, and progeny virus are produced. Because the entire sequence of the AAV genome is known, it is possible to specifically mutagenize any site in the DNA and assess the effects of the alteration on the replication phenotype. Using this approach, it has been possible to fairly rapidly map the AAV2 genome with respect to a variety of functions involved in the process of replication. One caveat is important with regard to the conclusions drawn. The details of the mechanism by which the AAV genome is rescued from the integrated state in the plasmid are unknown. It is possible that the AAV genome is first excised and then replicated. Alternatively, excision itself may be a function of the replication process as has been suggested by the "onion-skin" model for the rescue of integrated SV40 DNA (Botchan *et al.*, 1979). Thus, mutations that block AAV DNA replication may be at the level of rescue rather than replication, if indeed the two are separable phenomena.

With this reservation in mind two regions of the genome have been identified that are critical to DNA replication. The first is the inverted terminal repeat which is considered to function as the primer for AAV DNA replication (see Section IV,E below on DNA replication) (Samulski *et al.*, 1983; Senapathy *et al.*, 1984). A functional terminal repeat is required in *cis* for AAV DNA replication. The specialized sequence arrangement at the ends of AAV confers special properties. (1) Because of the palindromic nature of the terminal 125 bases, the DNA can hairpin to serve as a primer for DNA synthesis (Figs. 2 and 4). (2)

for repair exists because of the large deletion already present at the left end of pSM609.

The second region critical to AAV DNA replication is the ORF in the left half of the genome (Fig. 3). Deletions and nonsense mutations within this region block DNA synthesis (Hermonat *et al.*, 1984; Tratschin *et al.*, 1984a; Senapathy *et al.*, 1984). The 5′ ends of two AAV transcripts are within this region at map positions 5 and 19 (Fig. 3) (Lusby and Berns, 1982). The two transcripts are present in both spliced and unspliced forms, although the latter predominate (Laughlin *et al.*, 1979a). The unspliced forms would code for two polypeptides with overlapping amino acid sequences (the ORFs are in the same reading frame) of 68 and 44 kDa, respectively. Such polypeptides are present in infected cell extracts and are precipitated by antisera raised against oligopeptides synthesized to correspond to the appropriate DNA sequences in the ORF (M. A. Labow, K. Jones, B. Dunn, and K. I. Berns, unpublished observations). The sizes of the proteins estimated from mobility in SDS–acrylamide gels would suggest that they are translated from the unspliced forms of the transcripts. Because of the overlap in sequence it has only been possible so far to determine unambiguously that the product of the p5 transcript is required for DNA replication. Theoretically, it might be possible to directly demonstrate a requirement for the p19 transcript product by mutating the putative initiator codon to a nonmethionine codon, but this experiment has not yet been done. It is currently assumed that both of the polypeptides are required. Possible roles are discussed below in Sections IV,C and IV,E transcription and DNA replication, respectively. Smaller ORFs exist within this region. Whether they code for additional nonstructural proteins is unknown.

Mutations within the ORF on the right side of the genome do not affect the accumulation of the AAV duplex replicative intermediates (Hermonat *et al.*, 1984; Tratschin *et al.*, 1984a). However, the sequestration of mature virion linear single strands is affected. It is known (see below) that the ORF on the right side codes for the coat proteins. Thus, neither preformed capsids nor the individual coat proteins are required for AAV DNA replication, although one or both are required for accumulation of mature virion genomes, presumably by being involved in removal from the replication complex.

C. Transcription

Three basic AAV transcripts have been reported (Laughlin *et al.*, 1979b; Green *et al.*, 1980; Marcus *et al.*, 1981; Lusby and Berns, 1982). All are copied from the minus strand (Carter *et al.*, 1976). There is no

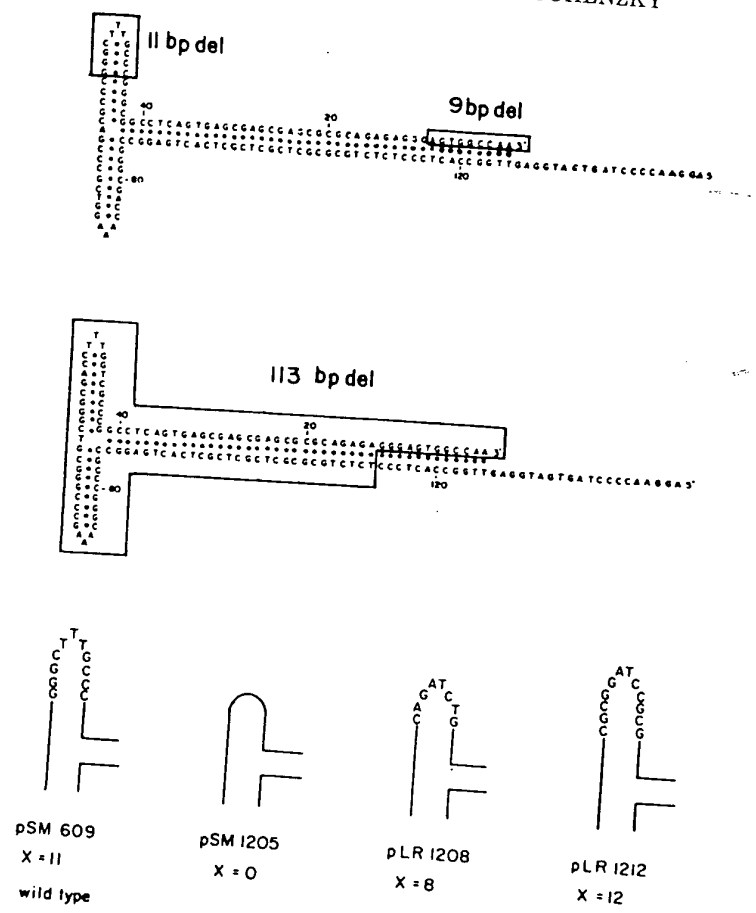

FIG. 5. Structure of terminal mutants of AAV. The top and middle figures correspond to the right and left ends of the mutants, respectively. The bottom figure shows the structures of various terminal mutants with respect to the 11b deletion in the internal palindrome shown in the top figure. All mutants contain both the 9b and the 113b deletions. (Reprinted with permission from Lefevre *et al.*, 1984, *Mol. Cell. Biol.* 4, 1416–1419.)

can be repaired. However, deletions that extend beyond the terminal repeat or for which a template does not exist should be lethal and indeed are in a cis-active fashion. Thus, deletion of an 11-base symmetrical sequence (forming the end of one of the crossarms of the T-shaped structure in Fig. 2, bases 47–57) from the right end of the viable deletion mutant pSM609 described above is lethal. No template

could then be processed to VP3, this does not seem likely. Rather it appears that VP3 is initiated at the first AUG in the ORF, but VP2 is initiated at the unusual codon ACG upstream from the first AUG (Becerra *et al.*, 1985). This may explain the relatively small amount of VP2 synthesized relative to VP3. In the case of VP1, it seems rather likely at the moment that a differently spliced 2.3-kb transcript is used to code for the protein. Several pieces of evidence are in accord with this notion. First, alternative potential splice donor and splice acceptor sites occur at predicted positions on the genome (Fig. 6). Second, the hypothetical mRNA resulting from the alternative splicing would contain an ORF in the appropriate frame large enough to code for VP1. Third, a large insertion was placed within the putative intron. If, and only if, the hypothetical alternative splicing event occurred could 2.3-kb AAV RNA have been produced. Such RNA was indeed seen when the experiment was done (C. Murphy, M. A. Labow, P. L. Hermonat, and K. I. Berns, unpublished observations). This is strong presumptive evidence for an alternative 2.3-kb mRNA as the source of VP1. Finally, mutants between map positions 48 and 53 result in a low yield of infectious particles, as might be expected for a mutant specific for VP1, which is a minor component of the virion

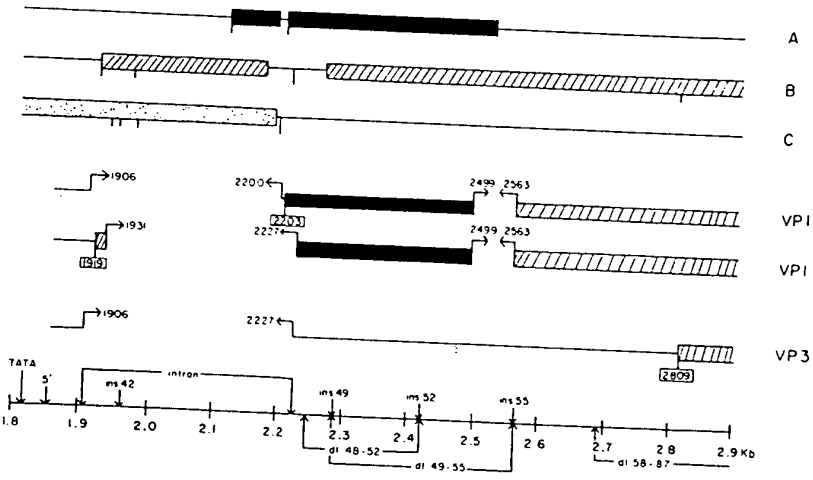

FIG. 6. Potential for alternative splicing of transcripts encoding the AAV capsid proteins. Lines A, B, and C represent the positions of open reading frames present in the area at 1.8 to 2.9 kb along the AAV genome. Lines VP1, VP1, and VP3 represent potential structures of mRNAs that could code for the proteins. Boxed numbers represent ATG start sites. Bent arrows represent known or postulated splice sites. Areas marked ins xx or dl xx-xx denote mutants known to exist in this area. (Reprinted with permission from Hermonat *et al.*, 1984, *J. Virol.* 51, 329–339.)

evidence for any transcription from the plus strand. The 5′ termini are at map positions 5, 19, and 40, respectively (Green and Roeder, 1980a,b; Lusby and Berns, 1982). All coterminate at map position 96 (Srivastava *et al.*, 1983), are capped (B. J. Carter, personal communication), and are polyadenylated (Carter, 1976). They have been dubbed the p5, p19, and p40 transcripts, respectively. All the 5′ termini are preceded by a TATA box 31 ± 1 bases upstream, and it is presumed that all of these TATA boxes denote independent promoters. Cloned segments of the genome containing only the p5 or the p40 promoters synthesize appropriate transcripts upon transfection into adenovirus-infected cells (Janik *et al.*, 1984; Labow *et al.*, 1986; Tratschin *et al.*, 1984b). The p19 promoter has yet to be tested in this respect. Additionally, the p40 promoter can function in an *in vitro* assay to initiate transcription (Green and Roeder, 1980b). By runoff assays no evidence has been found for any additional functional promoters, although additional potential TATA boxes exist within the DNA sequence (Lusby and Berns, 1982). A polyadenylation signal (AATAAA) exists approximately 21 bases upstream from the common terminus of the three transcripts (Srivastava *et al.*, 1983). Only one other such sequence exists in the genome and is found at map position 48 within the major intron. Only a small amount of transcript (less than 1%) is found that might correspond to termination near this site (M. A. Labow and K. I. Berns, unpublished observations). The significance of this minor fraction is unknown.

Both spliced and unspliced forms of all three major transcripts exist (Laughlin *et al.*, 1979; Green *et al.*, 1980; Marcus *et al.*, 1981). The intervening sequence removed is approximately 300 bases long from base 1906 to base 2228 on the genome (Srivastava *et al.*, 1983). Therefore, the sizes of the unspliced and spliced species of transcripts are p5, 4.2 and 3.9 kb, respectively; p19, 3.6 and 3.3 kb, respectively; and p40, 2.6 and 2.3 kb, respectively.

How many and which of the various AAV RNAs actually function as mRNAs *in vivo* is not completely clear. Only the p40 spliced 2.3-kb RNA, which constitutes approximately 90% of the total AAV RNA accumulated late in AAV-infected cells, has been isolated from polysomes (Green *et al.*, 1980). The p40 2.3-kb species is known to code for all three coat proteins, but the exact way in which this is accomplished is unknown (Jay *et al.*, 1981; Janik *et al.*, 1984; Becerra *et al.*, 1985). Can one RNA code for more than one protein directly? Several such cases are known for eukaryotic viruses, including the retroviruses. The case is further complicated by the fact that the ORF included in the major 2.3-kb spliced species is not large enough to code for VP1. Although the major 2.3-kb spliced species could code for VP2, which

effects of the AAV genome, plasmids in which the AAV genome is inserted into pBR322 are transfected into cells infected with helper adenovirus. Under these conditions, either 8-base inserts that cause nonsense mutations or large deletions within the left side ORF and that would affect only the protein(s) coded for by the p5 transcript or the proteins coded for by both the p5 and p19 transcripts depress accumulation of the major p40 2.3-kb spliced transcript (and the 2.6-kb unspliced species) to a very low constitutive level seen in the absence of helper adenovirus infection (Labow *et al.*, 1986). Similar effects are seen with plasmids containing only the p40 promoter (Innik *et al.*, 1984; B. J. Carter, personal communication; Labow *et al.*, 1986). This effect is not dependent on AAV DNA replication. It is possible to construct clones in which there are sufficiently large deletions within both terminal repeats (*ori*$^-$ mutants) that the AAV genome cannot be rescued and replicated, presumably because the terminal loop no longer hairpin to serve as primers for the initiation of DNA synthesis. When the internal sequences are intact, such clones accumulate normal levels of p40 transcripts, but not when the left-side ORF is inappropriately mutated. Since simple base inserts exhibit the effect, we conclude that one or more of the products of the left side of the AAV genome exert a positive regulatory effect on the expression of the AAV gene. This positive regulatory effect is complementable in trans as would be expected if the gene products were exerting the regulatory effect. Mutants of the type described above can be complemented by *ori*$^+$ mutants with large deletions in the right-side ORF. In such cotransfections there is a normal or greater than normal accumulation of intact 2.3-kb p40 RNAs transcribed from the left-sided mutant genome. Thus, the results are in accord with the prediction that the *rep* gene products might exert their effect(s) directly on the transcription process or on RNA stabilization. We now know that the former is the case (Labow *et al.*, 1986).

Mutations on the left side of the genome also depress the accumulation of either the p5 or the p19 transcripts. However, when expression is complemented certain mutations may actually increase such accumulation. When *ori*$^+$ left-side mutants with large deletions that include the p19 promoter are complemented by *ori*$^+$ genomes with right-side deletions in the capsid genes so that DNA amplification can occur, the p5 transcript from the left-side mutant accumulates to much greater than normal amounts, in fact to nearly the same extent that the p40 transcript from that genome accumulates. This would appear that there is a cis-active element in the AAV genome on the left side (between map positions 10 and 37) which exerts a negative regulation on at least the p5 promoter expression (we cannot yet about

capsid. One of these has a shortened VP1, but VP2 and VP3 are unaffected. Thus, it appears likely that the p40 transcript has two spliced forms.

Because both 68- and 44-kDa proteins which correspond to the left side ORF have been identified immunologically and because the left side of the genome is required to be intact in a trans-active manner for DNA replication to occur, it seems probable that both the p5 and the p19 transcripts or modifications thereof serve as messengers. Again, as pointed out above, the apparent molecular weights of the two *rep* proteins as determined from mobility in SDS–polyacrylamide gels are consistent with translation from the unspliced species of the p5 and p19 RNAs. Thus, whether a biological role exists for the spliced species or whether they are simply biological waste products as a consequence of containing splice donor and acceptor sites cannot be determined at this time. Also as stated above, a polyadenylation signal exists starting at base 2182. Under conditions where the normal polyadenylation signal was deleted some evidence exists for an RNA species polyadenylated at this position. Whether the species arises normally and, if it does what its function might be, are unknown (M. A. Labow and K. I. Berns, unpublished observations).

D. Self-Regulation of Gene Expression

Regulation of AAV transcription is one of the major functions supplied by helper virus coinfection. In the absence of a helper virus coinfection or the presence of functional adenovirus *E1a* and *E1b* genes in the integrated state (i.e., adenovirus-transformed cells) it is difficult, if not impossible, to detect AAV-specific transcription. As an aside, when plasmids containing AAV promoters, especially p40, are transfected (by means of the $CaPO_4$ coprecipitation technique) into cells lacking functional helper virus genes (Tratschin *et al.*, 1984b), or when AAV is infected into heat-shocked cells (M. A. Labow and K. I. Berns, unpublished observations), it is possible to detect low levels (approximately 2–5%) of AAV transcripts. With this type of exogenous regulation, a significant question arises as to whether the AAV genome functions passively with regard to regulation of the expression of its structural genes or whether it also plays a major active role in the regulation of this process. Evidence to be presented below quite clearly demonstrates that the latter is the case and that AAV quite clearly has a major role in the regulation of its gene expression by virtue of both cis-active regulatory sequences and trans-activation.

The left side of the AAV genome exerts a positive effect on accumulation of the p40 transcript. To demonstrate the self-regulatory

account and be able to explain the features of the mature DNA. Possibly the two outstanding features of the AAV genome are that the genome is a linear single polynucleotide chain and that the ends of the genome are inverted terminal repeats characterized by terminal palindromic sequences. A particular feature of the terminal repeats is the presence of sequence heterogeneity at both the very ends (i.e., 5′TTG, 5′TG, or 5′G) of the repeat and within the palindromic region (i.e., within the crossarms of the T-shaped structure shown in Fig. 2) (Fife *et al.*, 1977; Spear *et al.*, 1977; Lusby *et al.*, 1980). Any linear DNA faces special problems with respect to the replication of its ends. This is a consequence of the requirement of all known DNA polymerases for a primer with a free 3′ OH group as well as for a template. If the DNA goes through a circular replicative intermediate the problem is obviated, but, if it replicates via linear intermediates, specialized sequences are required at the termini to permit maintenance of the integrity of the terminal sequences. Thus, all linear viral DNA genomes that have been characterized have either some sort of inverted or natural terminal nucleotide sequence repeat (e.g., adenovirus and T-even bacteriophages, respectively) or palindromic terminal sequences (autonomous paroviruses) or a combination of the two (e.g., herpesviruses, poxviruses, and AAV). The model which has been derived for AAV DNA replication to a large extent, therefore, arises from consideration of the specialized sequence found at the termini.

The current model for AAV DNA replication is illustrated in Fig. 4. It is derived from a general model for the replication of eukaryotic DNA molecules that was first proposed by Cavalier-Smith (1974). Its special feature is that it requires a terminal palindromic sequence so that the DNA does not have to pass through multimeric replicative intermediates. In fact, AAV replicative intermediates are both unit length and oligomeric. The scheme is composed of the following steps.

1. The 3′ terminal repeat hairpins in the palindromic region serve as the primer for DNA replication. Because of the inverted terminal repeat the 3′ ends of strands of both polarities (plus and minus) are the same. Whether the termini of the incoming parental virion strands directly form the hairpin structures or first form single-stranded circles stabilized by base pairing between the inverted repeats is not known. The latter possibility is attractive for two reasons. The first is related to the fact that the AAV replicative intermediate is duplex. In order for the ends of the strands to be able to hairpin so that a subsequent round of synthesis may be initiated the duplex must be dissociated. If a panhandled circle stabilized by base pairing between the ends were to be formed, the first round of replication would be struc-

p19 expression because all of the mutants used have deleted the p19 promoter). We have termed this cis-active signal *dep,* or a depressor for transcription. It is most manifest when DNA replication occurs. Under *ori*$^-$ conditions with no DNA synthesis the accumulation of the p5 product is not so striking, but it is still equivalent to the accumulation of the p40 RNA species from the same genomes. Thus, it might be considered that the cis-active signal is a way to compensate for a significant increase in the number of potential templates for transcription. Under the conditions described above, when an *ori*$^+$ left-side deletion plasmid is complemented by an *ori*$^+$ right-side deletion plasmid, an actual decrease is seen in the amount of p40 RNA species from the genome with the right-side or capsid gene deletion relative either to transfection by that mutant alone or relative to the p40 species from the genome with the left-side *rep* gene deletion present in the complementation experiment. Therefore, it seems as though there may also be cis-active negative regulation of the capsid gene expression (p40) by a sequence on the left side of the genome. Whether or not the same cis-active signals are responsible for the negative regulation of p5 expression as are responsible for negative regulation of p40 expression is not yet known. There is evidently a fine modulation of gene expression, because early in infection the p5 and p19 transcripts are present in amounts at least equal to the level of p40 RNAs, but later, when the products of the p5 and p19 genes are present to positively regulate p40 expression, whatever negative signals there are must be more effective at depressing p5 and presumably p19 gene expression than p40 expression, which is required for structural protein synthesis. In summary, it seems clear that the regulation of AAV RNA metabolism is the consequence of a complex interaction between helper virus functions, and possibly host cell functions, and trans-active positive regulation by AAV gene products as well as modulated cis-active negative regulation by sequences within the AAV genome.

At least one host function has been directly implicated in AAV transcription. All of the promoters on the AAV genome correspond to those recognized by host cell RNA polymerase II and it is assumed that this is the enzyme responsible for AAV transcription. The p40 promoter was correctly recognized and served to initiate transcription *in vitro* using RNA polymerase II containing cell-free extract (Green and Roeder, 1980b).

E. DNA Replication

The mature virion genome is the end product of the process of DNA replication. Any model of DNA replication must, therefore, take into

ther, the model predicts that the orientation (flip or flop) of the terminus at one end of the DNA would not determine the orientation of the equivalent sequence at the other end of the molecule. Again, that is what is found *in vivo* (Lusby *et al.*, 1981). The agreement is somewhat indirect because it was originally impossible to purify AAV with a single orientation at either or both ends. This difficulty has now been overcome by virtue of the biological activity of clones of AAV DNA in pBR322. These clones by definition have a single orientation at both ends. The plasmid pSM620 has the terminal repeat in the flop orientation at both ends. When pSM620 is used to infect human cells in culture the progeny AAV genomes contain both orientations at both ends of the genome with equal frequency, directly demonstrating that the inversion is a consequence of the replication process (Samulski *et al.*, 1982). Use of pSM620 has also shed light on the origin of heterogeneity in the terminal two bases of the AAV genome (i.e., 50% of the 5′ termini are missing the terminal T and a further 15% are missing both the terminal T and the penultimate T). The insert in this plasmid is missing both 5′ terminal T's on both strands. The virion progeny strands have a majority of 5′ termini with T as the terminal base. What seems probable is that the putative nick site after base 125 is actually somewhat variable and can also occur after either base 123 or base 124. The complementary 3′ end can still hairpin to serve as a primer regardless of the terminal base. The first base or two polymerized will serve to replace either one or both missing bases.

The ability of AAV to repair short terminal deletions just described in the case of the terminal heterogeneity and the ability to repair much more extensive deletions within the terminal repeats described above in Section IV,B on genetics give some indication of the properties inherent in the palindromic inverted terminal repeats. This, together with the fact that it is the primer, make it clear that the T-shaped terminal structure plays a key role in the replication process. In the section on genetics a mutant with a deletion of bases 47–57 in the palindromic part of the terminal repeat (pSM1205) was described which rendered a previously viable deletion mutant nonviable (Samulski *et al.*, 1983). This was assumed to be a nonviable mutant because the original 113-base deletion in the terminal repeat at the other end of the genome had removed any potential template for repair of the new deletion. The 11-base symmetrical sequence which had been removed could have been required because of the sequence per se. However, another possibility was that the critical nature of the sequence was its contribution to the potential T-shaped conformation that the inverted repeat may form (Fig. 2). To assess the latter possibility

turally equivalent to the subsequent rounds, at the termini. The second reason is the capacity of the terminal repeats to self-repair large deletions at one end using the terminal repeat at the other end as a template. A likely model for the mechanism of such repair depends on the formation of such panhandled single-stranded circles.

2. Strand synthesis proceeds by elongation from the primer hairpin. No proteins, RNA primers, or Okasaki fragments have been found. Thus synthesis is unidirectional and asymmetric. When synthesis of a daughter strand is initiated in a duplex replicative intermediate, it proceeds by a single-strand displacement mechanism, similar to that observed for helper adenovirus DNA replication but not for helper herpesvirus DNA replication. These two aspects of the model are supported by several types of experimental results. Adeno-associated virus replicative intermediates from productively infected cells have been identified and can be chased into mature virion DNA (Straus *et al.*, 1976b; Hauswirth and Berns, 1979). The replicative intermediates have been isolated and characterized. They are cross-linked by hairpin structures at the termini and led to the original suggestion that the Cavalier-Smith model was applicable to AAV DNA replication (Straus *et al.*, 1976b). Pulse-labeling experiments *in vivo* have demonstrated that synthesis of both plus and minus strands start at the 5′ ends of the progeny strands and proceeds unidirectionally to the 3′ ends (Hauswirth and Berns, 1977).

3. In addition to simple strand elongation, the replication scheme accounts for replication and preservation of the termini. It is hypothesized that the parental strand in the replicative intermediate is specifically nicked at or near base 125 at a point opposite the original 3′ terminal base which is now incorporated into the progeny strand. The nick results in the transfer of the terminal 125 bases from the 3′ end of the parental strand to the 5′ end of the progeny strand, leaving a shortened parental strand with a gap at the 3′ end that can be repaired by synthesis using the free 3′ OH as a primer and the new 5′ overhang of the progeny strand as a template. Note that this transfer results in the inversion of the terminal 125 bases. Original base 1 at the 3′ end of the parental strand is base 125 at the 5′ end of the progeny strand after the transfer, while the orignal base 125 on the 3′ end of the parental strand is now the first base at the 5′ end of the progeny strand. Because bases 1–41 and 85–125 represent part of a perfect palindrome those sequences would not be affected by inversion. However, the two short internal palindromes from 42 to 62 and 64 to 84 are asymmetric with regard to the axis of the overall palindrome from 1 to 125. Thus, the model predicts the two sequences observed within this region of the terminal repeat in virion DNA (Lusby *et al.*, 1980). Fur-

perfect in every instance and significant numbers of defective genomes are generated. At least some of these genomes may be encapsidated to form defective-interfering particles which can inhibit normal AAV DNA replication but do not seem to be able to inhibit adenovirus DNA replication in the absence of added wild-type AAV genomes. Both double-stranded and single-stranded defective genomes have been identified. The single-stranded defective molecules have deletions of varying lengths of internal sequences but always retain the inverted terminal repeats. Two types of duplex defective molecules have been reported. Both are covalently cross-linked at one end and each contains sequences from either the left or the right end of the molecule only. Such duplex cross-linked molecules isolated from infected cells were reported to be cross-linked at the end generated from normally internal sequences in the full-length genome (Hauswirth and Berns, 1979), whereas duplex cross-linked molecules isolated from defective particles were reported to be cross linked via the hairpin form of the palindromic portion of the inverted terminal repeat (de la Maza and Carter, 1978, 1980). Whether the discrepancy in the site of the hairpin cross link is real has not been determined. Duplex molecules with a terminal hairpin within the internal sequences of the genome could be the consequence of a strand switch during the replication process. Such a switch could be to either the newly synthesized strand or to the displaced strand of the replicative intermediate. Evidence has been presented which would indicate that either new template is equally likely. Duplex molecules with a hairpin at the site of the terminal repeat could be visualized as the products of self-primed synthesis by genome fragments which contain the original 3′ termini. Generation of single-stranded molecules with extensive deletions may be the consequence of a deleted template generated by heterologous recombination (Senapathy and Carter, 1984) or a mistake during the replication process itself.

Sources of the specific proteins involved in DNA replication have not been directly determined. Putative replicative complexes isolated from HSV coinfected cells have been reported to contain the HSV DNA polymerase, while those isolated from adenovirus coinfected cells have been reported to contain the cellular polymerase (Handa and Shimojo, 1977; Handa and Carter, 1979). In light of the recent discovery of an adenovirus-coded DNA polymerase that is used to replicate adenovirus DNA (Enomoto *et al.*, 1981; Lichy *et al.*, 1982), the above results may be contradictory. To date early region *2b* which codes for adenovirus DNA polymerase has not been identified as a helper function required for AAV DNA replication. Comparably critical experiments have not yet been done with HSV. An additional question with regard to the

pSM1205 was modified by insertion of either a *Bam*HI linker (12-base symmetrical sequence) or a *Bgl*II linker (8-base symmetrical sequence) into the site of the deletion (Fig. 5). Either would result in the approximate restoration of the T-shaped conformation by permitting sufficient base pairing to reform the crossarm of the T disrupted by the original deletion. Both of these mutants permitted rescue of the genome from the plasmid, replication of the DNA, and production of infectious virions. When the progeny virion DNA was analyzed the altered sequences were present at both ends of the DNA and had undergone the inversion described above. Therefore, it would appear that the potential T-shaped conformation takes actual precedence over the specific sequence in this part of the terminal repeat in the case of DNA replication (Lefebvre *et al.*, 1984).

With this evident biological plasticity in DNA replication it is possible to test the potential relative efficiency of the wild-type versus mutant terminal sequences and to study to an even greater extent mutual interactions between the inverted terminal repeats. To investigate these questions a clone that was a chimera was constructed: the left terminal repeat had the wild-type sequence and the right terminal repeat the *Bgl*II mutant sequence (R. A. Bohenzky, R. B. Lefevre, and K. I. Berns, unpublished observations). The clone was biologically active and the progeny virion DNA was analyzed with respect to the following questions. (1) Was there transfer of a given sequence from one end to the other under conditions where both sequences were viable for replication? (2) Was there any preference for either sequence in the progeny? In repeated experiments all of the progeny virion genomes had the wild-type sequence at both ends. Thus, the wild-type sequence had a definite advantage *in vivo,* presumably at the level of the efficiency with which it was able to function in the replication process. The results represent essentially a form of gene conversion which could have occurred either as a consequence of recombination between the inverted terminal repeats or as a result of a DNA repair process via the panhandled single-stranded circular intermediates hypothesized to occur during AAV DNA replication (see Fig. 3). When the chimera was constructed in such a way as to reverse the orientation of the wild-type and mutant termini, however, no preference for either sequence was seen. This may indicate a polarity in the gene conversion event inherent either in the replication process or in the rescue/repair process. The latter possibility is preferred since experiments directly measuring the difference in replication efficiencies between wild-type and mutant termini show both sequences are similarly efficient.

As is the case with other viruses, AAV DNA replication is far from

SV40 to the system, which relieves the block for both adenovirus and AAV in a coinfection, or using an adenovirus host range mutant which can replicate in monkey cells. Such mutations occur in early region *2a*. As noted above, reports on the ability of temperature-sensitive *E2a* mutants to help AAV infection are somewhat in conflict, but at least one laboratory has reported results that are in parallel with those seen in monkey cells (Jay *et al.*, 1981). These also seem to function to change host cell specificity for mRNA translation by affecting the phosphorylation of the initiation factor 2. Also, as described above in Section IV,A on helper virus functions, the adenovirus VA RNAs are necessary for optimal AAV coat protein synthesis (Janik *et al.*, 1981).

The manner in which AAV uses essentially one sequence to code for three coat proteins is also of interest. It would now appear that VP1 is translated from an alternatively spliced transcript also of 2.3 kb. Although this transcript has not been directly isolated or demonstrated there are now several lines of evidence to support the notion. (1) There is not a sufficiently large ORF in the previously described 2.3-kb p40 transcript to code for a protein the size of VP1 (Srivastava *et al.*, 1983). (2) All coat proteins are translated from mRNA(s) of 2.3 kb (Jay *et al.*, 1981) (3) Even if the data cited in (2) cannot absolutely rule out a slight amount of unspliced 2.6-kb mRNA contamination, the unspliced species does not contain an appropriate ORF. (4) Alternative splice donor and acceptor sequences exist which could generate a 2.3-kb mRNA with an appropriate ORF (Srivastava *et al.*, 1983; Hermonat *et al.*, 1984). (5) Genetic data indicate the existence of the alternately spliced species (C. Murphy, M. A. Labow, P. L. Hermonat, and K. I. Berns, unpublished observations). (6) Evidence has been presented that the VP1 protein originates from a site that would correspond to the AUG in the ORF in the alternately spliced sequence (J. A. Rose, personal communication).

VP2 appears to be generated by a more novel mechanism. The first AUG in the ORF of the normally spliced 2.3-kb mRNA is 635 bases in from the 5′ end of the RNA. Its use would produce a protein of the size to correspond to VP3. It now has been reported that VP2 initiates at the codon ACG 195 bases upstream from the AUG used for VP3 (Becerra *et al.*, 1985). The use of ACG as an initiator codon has been reported before as a rare occurence. The AUG reported in the AAV sequence is in the optimal sequence environment to be able to function as an initator according to the rules established by Kozak (1980).

The extent of posttranslational modification of the coat proteins is uncertain. Several investigators have reported that the N-termini are blocked, apparently by acetylation. Additionally, multiple species of VP1 and VP3 can be separated by SDS–polyacrylamide gel elec-

DNA polymerase involves whether a second polymerase might be involved in the "repair" synthesis of the termini. Other proteins which may well be directly involved in the replication of AAV DNA include the putative nickase that transfers the hairpin from parental to progeny strand and a protein which would separate the duplex ends of the replication intermediate to allow hairpin primer formation. Whether either or both of these may be coded for by the AAV *rep* genes is still an open question. Additional uncertainties include the role of the 25-kDa polypeptide coded for by adenovirus early region *4*, which seems to be required for AAV DNA replication, and whether there may be any role for topisomerase in the process. In summary, the enzymology of AAV DNA replication remains very much a black box.

F. Protein Synthesis

With the identification of two polypeptides coded for by the *rep* genes on the left side of the AAV genome it has been possible to discern an apparent temporal order in the synthesis of the AAV proteins. In cell culture the 44-kDa polypeptide corresponding to the p19 transcript can first be detected 6 hours postinfection at an moi of 20 $TCID_{50}$/cell (M. A. Labow, K. Jones, B. Dunn, and K. I. Berns, unpublished observations). The 68-kDa polypeptide coresponding to the p5 transcript is not seen until 13 hours postinfection when the 44-kDa protein is still present. By 20 hours the 44-kDa protein is no longer present but the 68-kDa protein remains. By this time the coat proteins also have appeared. A simple but very tentative model based on the transcriptional regulation data and the genetic data would suggest that the 44-kDa protein allows expression of the 68-kDa protein, which in turn is involved in DNA replication more directly and potentially in the positive and/or negative regulation described in Section IV,C on transcription. Determination of specific functions of the *rep* gene proteins awaits development of suitable expression vectors so that sufficient amounts of the proteins can be isolated for biochemical studies.

Synthesis of the *rep* gene proteins seems to differ from that of the capsid gene proteins. The basis for this conclusion rests primarily on experiments using adenovirus early region *2a* mutants as helper and on experiments in monkey cells with a human adenovirus helper (McPherson *et al.*, 1982). Monkey cells are not normally permissive for human adenovirus. The defect appears to be at the level of translation of at least some of the structural proteins. Likewise, under the conditions described above AAV DNA synthesis occurs, but infectious viruses are not produced. The defect can be overcome by either adding

over 90 passages. Approximately 30% of these clones were positive for AAV rescue and they have been used to ask a variety of questions regarding the latent state of AAV. All clones made at passage 39 postinfection with AAV2 have remained positive for rescue over 150 passages (Hoggan *et al.*, 1972; Berns *et al.*, 1975; M. D. Hoggan, personal communication).

A. DNA Structure of the Latent State

The presence of AAV DNA in the clones was detected and quantitated by liquid hybridization analysis (Berns *et al.*, 1975). The level of AAV DNA present in these clones corresponded to 3–5 genome copies per diploid amount of cell DNA. KB cells latently infected with AAV1 were similarly quantitated and found to contain 4–6 copies per diploid amount of cell DNA (Handa *et al.*, 1977).

When total KB cell DNA was denatured and reannealed in a way that would allow formation of high-molecular-weight networks between highly repetitive elements within the genome (Varmus *et al.*, 1973), greater than 90% of the AAV DNA remained associated with the network, suggesting a covalent linkage between the AAV and cellular DNA (Handa *et al.*, 1977). Southern blot analysis of Detroit-6 cellular DNA from AAV2-infected cells also showed covalent linkage between viral DNA and cellular sequences of high molecular weight (Cheung *et al.*, 1980). Analysis of late passage (greater than 100) cells showed that low-molecular-weight AAV DNA was also present and was indistinguishable from duplex viral DNA. The origin of this low-molecular-weight form is unclear, as it was not present in early passage (8–9) cells.

Southern blot analysis of the Detroit-6 clones using restriction endonucleases answered some detailed questions about the integrated state. Digestion with enzymes that do not cleave AAV DNA (*Bgl*II, *Pvu*I, *Hpa*I) lowered the molecular weight of the provirus while still maintaining it as a single band, consistent with covalent linkage between the AAV DNA and cellular DNA at a single site of integration. Digestion of cellular DNA with enzymes that cleave AAV DNA once (*Bam*HI, *Hin*dIII) produced monomer-length bands suggestive of a tandem repeat arrangement of the multiple copies of viral DNA. These bands can be visualized using probes specific for either the right or the left sides of the viral genome. Digestion with enzymes that cut AAV DNA at multiple sites (*Hinc*II, *Pst*I) mapped the point of linkage between the viral and cellular DNA to the termini of viral DNA. Analysis of several clones revealed that the linkage is always through the termini, although the location in cellular DNA may be different

trophoresis (McPherson and Rose, 1983). The assumption is that the multiple species represent a heterogeneity in some sort of posttranslational modification, although there has been no evidence for either glycosylation or phosphorylation.

V. Latent Infection

When hamsters are inoculated intranasally with AAV, they do not develop antibody to AAV until after they are superinfected with adenovirus (R. W. Atchison, personal communication). This seroconversion takes place even if the superinfection is delayed several months. The defective nature of AAV poses an interesting evolutionary question. How does a virus that is completely dependent on coinfection with a helper virus maintain its biological continuity in the absence of that helper virus?

The initial answer to that question came from a program conducted by the National Institutes of Health to screen cell culture lots to be used for vaccine production for the presence of persistent viral infection. Whereas none of the cell lots were positive for AAV structural antigens initially, infection of the cells with adenovirus led to infectious AAV production in approximately 20% of African green monkey kidney cell lots and 1–2% of human embryonic kidney cell lots (Hoggan, 1970; Hoggan *et al.*, 1972). These data suggested that AAV could exist in some latent state in cells that were not coinfected with helper virus and that superinfection with adenovirus could rescue AAV from that latent state.

The relative ease with which one could convert a mammalian tissue culture cell, normally nonpermissive for AAV growth, to a cell capable of growing AAV, simply by addition of a helper virus, suggested a unique tissue culture model that could be used to study the intracellular mechanisms of latent viral infection. Such a model was set up using the human cell line Detroit-6 established by Berman *et al.* (1955). Detroit-6 cells were infected with purified stocks of AAV1H, AAV2H, or AAV3H (Hoggan *et al.*, 1972). When infected at high multiplicities, the cells remained positive for AAV structural antigens for approximately five passages. After that, AAV antigens could only be seen if the cells were challenged with adenovirus. Addition of 5-iodo-2′-deoxyuridine (IUdR) did not activate the AAV by itself but, when added with adenovirus, it increased the number of cells that rescued AAV. Similar effects were seen with IUdR and 5-bromo-2′-deoxyuridine (BUdR) on KB cells latently infected with AAV1 (Handa *et al.*, 1977). The former cultures remained positive for AAV rescue

partial rounds of DNA synthesis. Uninfected cell extracts are capable of synthesizing AAV RF from viral strands *in vitro,* suggesting such synthesis is possible *in vivo* (W. W. Hauswirth and K. I. Berns, unpublished observations). When HeLa cells are infected with AAV that has been radiolabeled with ^{32}P and density-labeled with BUdR, one can detect intermediate density material on cesium chloride gradients from DNA extracts of these cells (R. A. Bohenzky and K. I. Berns, unpublished observations). Such intermediate density material is preferentially hairpinned as assayed by denaturation followed by S1-nuclease digestion. Sucrose gradient analysis indicates that the material is low molecular weight, cosedimenting with AAV monomer duplex DNA. These data suggest that AAV DNA undergoes some metabolism in the absence of helper virus. Unfortunately, this metabolism takes place at far too low a level to analyze the structure with certainty. It is also not known whether this represents an intermediate in the integration process or whether this metabolism creates a "dead-end" structure having nothing to do with the latent state.

The cloning of AAV into bacterial plasmids (Samulski *et al.,* 1982; Laughlin *et al.,* 1983) has allowed a variety of physical manipulations aimed at understanding the genetics of AAV-related events. It has been possible to create an AAV genome with a dominant selectable marker and make virus stocks from this construct (Hermonat and Muzyczka, 1984; Tratschin *et al.,* 1985). These virus stocks can be used to infect cells in the absence of helper virus, and cells in which AAV DNA has been stably integrated can be selected for on the basis of the marker. The frequency of transduction varies directly with the incubation time prior to selection. Waiting for 1 week before selection can increase the transduction frequency to 10%. The frequency of transduction was also dependent on the cell line used, with Detroit-6 cells being the best.

Southern blot analysis of the cellular DNA shows that, in many respects, the integrated provirus of these transducing vectors resembles that of wild-type AAV (Tratschin *et al.,* 1985; P. Hermonat, P. Collis, and N. Muzyczka, personal communication). In both cases integration takes place at different locations within the cellular genome. Also tandem repeats are seen in both cases, although in the case of the transducing vector, single copy co-integrates are also seen. With this system, increased time of incubation prior to selection increases the proportions of cell clones with tandem repeats (P. Collis and N. Muzyczka, personal communication). Finally, in both cases, deletions are seen in some of the copies of integrated DNA.

The similarities between wild-type integration and vector integration suggest that this system may be a valid way of studying the

(Berns *et al.*, 1982). These digests with multiple-hit enzymes also showed the existence of unique fragments that correspond to deletions of or insertions into viral DNA in some of the integrated copies (Cheung *et al.*, 1980). Passage of the cells in culture did not change the restriction pattern of integrated viral DNA for any of the internal sites, but digestion with an enzyme that is specific for the termini (*Sma*I) gave a different pattern between early and late passage cells.

In summary, it appears that AAV DNA integrates into cellular DNA as a tandem repeat of several copies joined to cellular DNA through the termini. At least some of these copies may contain internal rearrangements but, with the possible exception of the termini, no active rearranging is seen after integration.

Nothing is yet known about the fine structure arrangements at the junction between viral and cellular DNA. It is not known whether the cellular DNA contains homology with AAV in the flanking region or if the flanking region is duplicated upon integration. The termini of AAV exist in different sequence orientations and it is not known if all these orientations are present in the integrated state. The answer to these questions awaits the cloning of the integrated provirus into a bacterial vector and subsequent sequencing across the viral–cellular junctions.

B. Mechanism of Integration

It is generally held that an understanding of the integration mechanism of AAV is linked to an understanding of the mechanisms of illegitimate recombination. Several kinds of experiments have been done to elucidate this mechanism. They include (1) the fate of AAV after infection of nonhelper virus-infected cells, (2) the use of selectable markers spliced into the AAV genome, (3) mutation of the AAV genome in these selectable marker systems, and (4) assays of recombination between AAV and other defined DNAs.

When cells are infected with AAV in the absence of helper virus, AAV particles can still adsorb and penetrate the cell membrane (Rose and Koczot, 1972; K. I. Berns and S. Adler, unpublished observations). They are transported to the nucleus and uncoated there. Adeno-associated virus DNA in the form of monomer duplex can be detected by Southern blot analysis for over 1 week postinfection (Z. Grossman, E. Winocour, and K. I. Berns, unpublished observations). Quantitative hybridization analysis using either filter hybridization (Rose and Koczot, 1972) or Southern blots (Laughlin *et al.*, 1982) shows no net AAV DNA synthesis in the absence of helper virus.

These techniques however, may not be sensitive enough to detect

differences (Grossman *et al.*, 1985). In DNA cotransfection experiments, recombination may take place between any point in the SV40 genome and any point in the AAV genome. Most of the recombinants from virion coinfections, however, contained copies of AAV termini integrated near the SV40 origin of replication. A deletion of SV40 sequences occurred at the point of integration. Multiple copies of the AAV termini and SV40 *ori* were present in a tandemly repeated arrangement. Sequence analysis of the junctions showed no obvious homology between AAV and SV40 at the junction other than the "patchy" homology seen in other types of SV40 recombinants (Gutai and Nathans, 1978a,b). This type of structure was not seen in virion coinfection experiments between SV40 and polyoma (py) (Z. Grossman and E. Winocour, personal communication).

The type of recombinant structure seen with AAV-SV40 virion coinfection can also be seen in DNA cotransfection experiments between SV40 and bacteriophage ϕX174 when linearized SV40 DNA is added as a third DNA in the transfection mixture (Dorsett *et al.*, 1985; Dechaite *et al.*, 1985). The reason for such similarity between these two very different experiments is unclear.

It should be noted that these recombinants were isolated as virion plaques in high-density platings of SV40. This procedure places two constraints on the recombinants isolated: (1) they must contain an SV40 origin of replication, and (2) they must be of a size that can be packaged into SV40 virions. The first constraint means that any recombinant that inactivates the SV40 origin would not be scored or isolated. The second constraint means that recombinants with full-length copies of AAV and SV40 would not be scored or isolated. The recombinants isolated, therefore, may represent either a subset of recombination events that produced packageable recombinants or a subset of recombinants that had undergone secondary recombination in order to reduce them to a packageable size. It may not be possible therefore, to deduce an integration mechanism from the structures thus far analyzed. Recombinant structures need to be isolated directly from the cell either by cloning low-molecular-weight DNA into bacterial vectors or by developing a system in which AAV integration into episomal shuttle vectors can be scored.

In summary, the mechanism by which AAV DNA integrates into chromosomal DNA remains unknown. The fact that AAV can integrate into different sites in the chromosome and the fact that no major homology is seen in AAV–SV40 recombinants suggest that the integration event involves nonhomologous recombination. The fact that there are different types of recombinant structures in virion coinfec-

genetics of AAV integration. Vectors have been made that substitute the neomycin resistance gene for either the *rep* or the *cap* region of the AAV genome (P. Hermonat, P. Collis, and N. Muzyczka, personal communication). Both the frequency of transduction and the structure of the provirus resemble bona fide AAV integration with either of the mutants. This suggests that no AAV-encoded protein is required for the integration event.

Detailed understanding of the mechanisms of integration require not only analysis of the integrating DNA but also analysis of the recipient DNA. As a result of this reasoning, attempts have been made to develop an assay system for recombination between AAV and some other defined DNA.

One such system has been reported which is based on the *in situ* plaque hybridization assay of nonhomologous recombination that is seen in monkey cells transfected with Simian virus 40 (SV40) and some nonreplicating DNA (Winocour and Keshet, 1980; Dorsett *et al.*, 1983). In this system, SV40 DNA (recipient) and a nonreplicating DNA (donor) are cotransfected into cells permissive for SV40 replication. The tansfected cells are then replated in an excess of untransfected cells and the plates are allowed to incubate until viral plaques are formed. The plaques are then transferred to nitrocellulose filters and hybridized with radiolabeled probe for either SV40 DNA or the nonreplicating DNA. The ratio of plaques containing donor DNA to those containing SV40 DNA is thus determined and represents a nonhomologous recombination frequency between donor and recipient DNAs. The recombinant plaques can be isolated and amplified, and their DNA used to analyze the recombinant structures.

When AAV DNA as the donor was cotransfected with SV40 DNA, nonhomologous recombination was seen at frequencies similar to those seen when other nonreplicating DNAs are cotransfected with SV40 DNA (Grossman *et al.*, 1984). The kinetics of this recombination event suggest that it takes place prior to the onset of SV40 DNA replication.

When AAV and SV40 were introduced into the cells as virions in a coinfection, the frequency and kinetics of the recombination events remained unchanged. A striking difference was seen however, when the structures of the recombinants were analyzed. Relative to their size, the termini of AAV were overrepresented in recombinants isolated from virion coinfection. In fact, greater than 90% of the recombinants contained terminal sequences. This was not seen with recombinants isolated from DNA cotransfections.

Detailed analysis of the recombinant structures revealed further

creased the percentage of rescuable clones. It is interesting to note that, although there have been tandemly repeated integrates that are nonrescuable, to date no rescuable cell clone has been found that is not, at least partially, tandemly repeated. Why a tandem repeat structure is required for rescue is not clear. It may be that several gene copies are required to overcome the effects of terminal or internal deletions in the integrated structure.

The cell clones which are rescuable have been used in experiments aimed at determining the mechanism of rescue. When latently infected KB cells were superinfected with adenovirus type 31 (Ad-31), infectious AAV1 could be detected as early as 18 hours post superinfection (Handa *et al.,* 1977). This contrasts with AAV1/Ad-31 coinfection, in which infectious AAV1 can be detected as early as 14 hours postinfection. Adeno-associated virus capsid antigen can be detected 10 hours post superinfection, and estimates of the fraction of cells making AAV antigen vary from 50 to 90% (Hoggan *et al.,* 1972; Handa *et al.,* 1977).

The addition of halogenated nucleotides enhanced the induction of AAV from either KB cells or Detroit-6 cells (Hoggan *et al.,* 1972; Handa *et al.,* 1977). The fraction of cells making AAV antigen increased with increasing concentration of BUdR or IUdR, but the 16-hour lag time remained unaffected. This enhancement was independent of irradiation by either visible or ultraviolet light. No induction of AAV as measured by antigen production was seen with the addition of halogenated nucleotides alone. Adenovirus was always required. However, partial rescue events, such as DNA replication, have not been assayed.

The dependence on adenovirus for rescue suggests the possibility of finding specific adenovirus gene products that are required for the rescue process. Many adenovirus mutants have been screened and several are defective for AAV DNA replication in coinfection experiments (Ostrove and Berns, 1980; Myers *et al.,* 1980; Myers and Carter, 1981; Janik *et al.,* 1981; Richardson and Westphal, 1981; Laughlin *et al.,* 1982; Carter *et al.,* 1983). Only one mutant has been found thus far that allows AAV DNA replication in coinfections but does not rescue AAV from latently infected Detroit-6 cells (Ostrove and Berns, 1980). This mutant, *hr 6,* is a mutant in early region lb (*Elb*) of Adenovirus type 5 (Ad-5) (Graham *et al.,* 1978). This is one of the transforming genes of adenovirus, but its function in AAV rescue remains unclear.

The rescuability of AAV from clones in bacterial plasmids (Samulski *et al.,* 1982; Laughlin *et al.,* 1983) suggests a system analogous to rescue from chromosomal DNA. Indeed, infectious AAV was only pro-

tion and DNA cotransfection experiments suggests more than one recombination pathway exists. It is the pathway used in virion coinfections that is more likely to be biologically relevant to AAV.

No AAV gene products appear required in the integration process, but it is possible that cis-active sequences in the genome are necessary. Both the genetic analysis of integration as well as the structures of AAV–cell and AAV–SV40 recombinants suggest that those cis-active sequences are in the AAV termini.

C. Rescue

Definition of a latent infection depends upon subsequent rescue of the organism from the latent state. Indeed in the case of AAV, latent infection was discovered by rescuing AAV following superinfection of cells by adenovirus (Hoggan *et al.*, 1972; Handa *et al.*, 1977). When single-cell clones are made from cell cultures infected with AAV in the absence of helper virus, approximately 30% of the clones are latently infected as determined by rescue (M. D. Hoggan, personal communication). Since rescue requires all aspects of AAV replication and since it has been noticed that some integration events result in AAV DNA rearrangement (Cheung *et al.*, 1980; P. Hermonat, P. Collis, and N. Muzyczka, personal communication), the question arises: do rescuable cell clones represent a subset of integration events? Conversely, is there nonrescuable integration? These questions have been approached in two ways.

First, cellular DNA from some of the cell clones that was negative for rescue was blotted and hybridized to AAV probe. Several of the clones contain AAV sequences (B. A. Beeler, T. Stukenberg, R. A. Bohenzky, and K. I. Berns, unpublished observations). At least one of these clones was rescuable at the DNA level when challenged with adenovirus. Unfortunately, these cells were cloned several passages postinfection so that it is not known how many discrete integration events are represented in these clones. This work needs to be repeated with cells cloned immediately after infection.

Second, cell clones isolated from infection with an AAV–neomycin vector can be tested for rescue at the DNA level (Hermonat and Muzyczka, 1984; Tratschin *et al.*, 1985). The majority of these clones were nonrescuable when challenged with adenovirus. An interesting correlation was noted when these clones were analyzed by Southern blot. Rescuability correlated well with the presence of tandem repeats as mentioned above. Increased incubation time prior to selection in this system increased the occurrence of tandem repeats. This also in-

how inhibitory to adenovirus replication. Indeed, it is the process of adenovirus DNA replication that is inhibited by AAV (Carter *et al.*, 1979; Lauglin *et al.*, 1979), suggesting a competition for factors involved in DNA synthesis. Although the adenovirus gene products necessary for adenovirus DNA replication (Ikeda *et al.*, 1981) are different from the adenovirus gene products necessary for AAV DNA replication (Richardson and Wesphal, 1981; R. J. Samulski and T. Shenk, personal communication), it is possible that some competition exists for cellular factors involved in DNA replication. In this scenario, AAV would be behaving as a DI particle of adenovirus.

Although Herpes simplex virus can serve as a helper virus for AAV (Buller *et al.*, 1981), there has been no report of AAV inhibiting Herpes simplex virus replication. In fact, it has been shown that AAV alone does not inhibit Herpes simplex replication in monkey cells (Parks *et al.*, 1968) but can inhibit Herpes simplex if a third virus, the simian adenovirus SV15, is added. The SV15 alone also does not inhibit Herpes simplex. The mechanism by which this inhibition takes place is unknown. The fact that AAV alone does not inhibit Herpes simplex replication is not inconsistent with the idea that adenovirus and AAV compete for cellular factors. Herpesviruses encode more of their own functions required for DNA replication and rely much less on the host cell than adenoviruses. There may be no such competition between Herpes simplex virus and AAV in lytic infection.

It is interesting to note that AAV can inhibit the replication of one virus that is not a helper virus. Coinfection of AAV and SV40 into monkey cells results in inhibition of SV40 plaque formation (Casto *et al.*, 1967b). The fact that SV40 does not help AAV DNA replication (R. J. Samulski and N. Muzyczka, personal communication) suggests that this inhibition may take place at the level of SV40 gene expression. Recent experiments are consistent with this idea. A gene transfer system has been developed using a murine melanoma cell line as a recipient (Graf *et al.*, 1984). Using the pGCcos3neo vector (G. Crouse, NCI Frederick Cancer Research Facility, Frederick, Maryland) which contains a fragment of the pSV2neo vector constructed by Southern and Berg (1982), one can stably transform cells, at a frequency of approximately 10^{-2}–10^{-3}, to a phenotype in which they are resistant to the antibiotic geneticin. When plasmids containing AAV are added with pGCcos3neo in these transfection experiments, a decrease in the number of resistant colonies is seen. This inhibition of transformation is at the level of 95% or greater (M. A. Labow, L. H. Graf, and K. I. Berns, unpublished observations). Similar results have been seen in HeLa cells, an immortalized human cell line (B. J. Carter, personal communication), and murine Ltk^- cells (M. A. Labow, L. H. Graf, and

duced from the clone when cells were coinfected with adenovirus. Infection with *hr 6* did not rescue AAV DNA from the clone either (R. J. Samulski and N. Muzyczka, personal communication).

Herpes simplex virus can also rescue AAV from both latently infected KB cells (C. A. Laughlin *et al.*, 1986) and cloned plasmid DNA (M. A. Rayfield and K. I. Berns, unpublished observations). It appears, however, to be more efficient at rescue of AAV DNA than at rescue of infectious virus. No genetic analysis of herpes mutants on AAV rescue has been reported.

The possible role of AAV gene products in rescue is a complicated one. The requirement for AAV DNA replication in the assay of the rescue process has made it impossible to separate the rescue process from replication, if indeed the two processes are separable. Recently, assays have been developed that detect a rescue event independent of replication. When AAV-containing plasmids were transfected into HeLa or KB cells in the absence of adenovirus, the presence of monomer duplex AAV DNA was detected at low levels (H. J. Gottlieb and N. Muzyczka, personal communication; R. J. Samulski and T. Shenk, personal communication). This species was also seen when a *rep* mutant of AAV was used. Furthermore, digestion of the product DNA with the endonuclease *Dpn*I, an adenine methylation-requiring nuclease, indicated that the species was not a product of replication (R. J. Samulski and T. Shenk, personal communication). This result was consistent with the above mentioned data from latently infected Detroit-6 cells in which monomer duplex DNA was seen at low levels in these cells at high passage numbers (Cheung *et al.*, 1980). Whether this monomer duplex DNA is a true intermediate in the rescue process or a structure not related to rescue is a question that remains to be answered.

An *in vitro* system has been developed that produces monomer duplex AAV DNA from cloned plasmids. Cell-free extracts of uninfected HeLa cells had this activity, but more activity was found in extracts from adenovirus-infected cells (H. J. Gottlieb and N. Muzyczka, personal communication).

The ability to rescue AAV DNA from uninfected cells and uninfected cell extracts raises the question of the contribution of cell physiology and cell type to the rescue process. Variation in rescue efficiency with adenovirus among cell types has been noticed in our laboratory (R. J. Samulski, P. L. Hermonat, M. A. Labow, R. B. Lefebvre, R. A. Bohenzky, N. Muzyczka, and K. I. Berns, unpublished observations). KB cells seem to rescue best and Detroit-6 cells seem to rescue worst. This would indicate that care should be taken when comparing experiments performed in different cell lines.

In summary, the biology of AAV has allowed it to be used as a

uniquely simple model for latent viral infections in animal systems. The 10–30% frequency with which latent infection can be established and the 50–90% frequency with which latent infection can be rescued compares favorably with other model systems, including bacteriophage λ. While we have learned much about the general nature of AAV latency, many of the mechanistic details have yet to be elucidated.

VI. Inhibition of Other Viruses

The effect of helper viruses on the growth of AAV is well established. Due to the defective nature of AAV, it is completely dependent on helper virus functions for its own growth. It is also interesting, however, to consider the possible effects of AAV on the helper virus. It has long been established that, in a plant virus system, tobacco necrosis virus helps a defective virus, tobacco necrosis satellite virus. Conversely, the satellite virus inhibits the replication of tobacco necrosis virus (Kassanis, 1962). A similar phenomenon is observed with AAV. Adenovirus helps the growth of AAV but, conversely, AAV inhibits the growth of adenovirus.

It is also interesting to note that this observation can be extended beyond the lytic cycle of adenovirus replication. Both adenoviruses and herpesviruses are able to transform cells in tissue culture (Freeman *et al.*, 1967a; Williams, 1973; Duff and Rapp, 1971a,b). Additionally, adenoviruses are oncogenic in certain animal models (Trentin *et al.*, 1962; Huebner *et al.*, 1965) and Herpes simplex viruses have been associated with human neoplastic disease of the female genital tract (Naib *et al.*, 1969; Rawls *et al.*, 1969; Aurelian *et al.*, 1970; Schwartz and Naftolin, 1981). Adeno-associated virus inhibits the oncogenicity of these viruses both in tissue culture and in animal model systems.

A. Inhibition of Lytic Infection

The ability of AAV to inhibit adenovirus production has been measured in a variety of systems (Hoggan *et al.*, 1966; Casto *et al.*, 1967a,b; Blacklow *et al.*, 1967; Parks *et al.*, 1968; Carter *et al.*, 1979). This inhibition phenomenon mimics helper function with regard to species specificity. Whereas human AAV will replicate in cells of other species if the adenovirus helper is of that same species, human AAV will inhibit adenovirus replication across the same species boundaries. In other words, human AAV inhibits human adenovirus in human cells

K. I. Berns, unpublished observations). Cotransfection with mutant AAV plasmids mapped the region responsible for inhibition to the *rep* region of the AAV genome (M. A. Labow, L. H. Graf, and K. I. Berns, unpublished observations). Since the geneticin resistance gene being scored for is under the control of the SV40 early promoter/enhancer in the pGCcos3neo vector, the possibility exists that an AAV *rep* gene(s) can inhibit expression of genes controlled by the SV40 early promoter. Experiments are currently underway to test this hypothesis.

These data on the inhibition of other viruses by AAV in tissue culture suggest a possible role for AAV during viral infections *in vivo*. Is it possible that AAV protects a host from infection by either adenoviruses or papovaviruses? This question has been addressed directly in a murine model system. When pregnant mice are infected with AAV1 and murine adenovirus (MAV), the AAV is transmitted to the progeny transplacentally but the MAV is not (Lipps and Mayor, 1980). Normally MAV produces a lethal infection when inoculated into newborn mice (Hartley and Rowe, 1960), but when newborn mice that had acquired AAV transplacentally were challenged with MAV, a decrease in MAV lethality by three orders of magnitude was observed (Lipps and Mayor, 1982). This was not seen when pregnant mothers were infected with adenovirus alone. Furthermore, the newborn mice were nursed by foster mothers that had not been infected with MAV, ruling out passive transfer of secretory antibody as an explanation of this result.

These results suggest an interesting role for AAV in the infectious process. Adeno-associated virus has not been associated with any disease itself. It may be that the ecologic role of AAV is to depress the pathogenicity of other lytic viral infections. Additionally, AAV may play a role in inhibiting the oncogenicity of other viral infections. These data are discussed below.

B. Inhibition of Oncogenicity

The existence of adenovirus helper functions for AAV and the ability of AAV to inhibit the lytic cycle of adenovirus growth suggest the possibility that AAV may also inhibit the oncogenicity of adenovirus. Although no human tumors have been associated with adenovirus, infection of neonatal Syrian hamsters (*Mesocricetus auratus*) with certain serotypes of adenovirus resulted in the appearance of undifferentiated sarcomas occuring approximately 35–80 days postinoculation (Trentin *et al.*, 1962; Huebner *et al.*, 1965). The degree to which adenoviruses are oncogenic, as measured by the fraction of hamsters developing tumors as well as the mean latent period before tumor devel-

opment, varies with the serotype of adenovirus used. Adenovirus type 12 is highly oncogenic, whereas adenovirus type 5 does not cause tumors. Nevertheless, all serotypes of adenovirus can transform rodent cells *in vitro* regardless of their oncogenic potential *in vivo* (Freeman *et al.*, 1967a–c; van der Eb *et al.*, 1979).

The discovery of AAV particles in adenovirus preparations (Archetti and Bocciarelli, 1963; Atchison *et al.*, 1965; Melnick *et al.*, 1965; Hoggan *et al.*, 1966) led to a series of experiments aimed at determining the effect of AAV on the oncogenicity of adenovirus type 12. When AAV1 was injected into newborn hamsters along with adenovirus-12, a reduction from 60 to 20% in the fraction of animals developing tumors was seen (Kirschstein *et al.*, 1968). Additionally, the mean latent period before tumor development increased from 45 to 64 days. These experiments were repeated using another oncogenic strain of adenovirus, type 31 (Mayor *et al.*, 1973). In this case no inhibition of oncogenicity was seen when the viruses were coinfected into newborn hamsters, but inoculation with AAV1 24 hours prior to inoculation with adenovirus-31 resulted in a decrease in tumor development. This decrease was not seen when SV40 was used as the oncogenic virus instead of adenovirus.

Inoculation of pregnant mothers with AAV did not protect the offspring from tumor development when inoculated with adenovirus-31 shortly after birth. In fact, in the case of female progeny, the fraction of animals developing tumors increased to 100%. The reason for the increase in tumor incidence in female progeny is unknown. These results differ from the experiments previously mentioned in which transplacental transmission of AAV protected progeny from lytic adenovirus infection (Lipps and Mayor, 1982), suggesting a difference in the mechanism of AAV protection from lytic and oncogenic adenovirus infections.

Gilden *et al.* (1968a) found no protection from adenovirus-12 tumor induction by AAV. This paradox was explained by low infectivity of the AAV preparation used (Gilden *et al.*, 1968b). Recent data, however, suggest that AAV infectivity, as defined by virus titer, is not a prerequisite for the inhibition of adenovirus oncogenicity. When purified DI particles of AAV were injected into newborn hamsters, a decrease in the fraction of animals developing tumors after subsequent inoculation with adenovirus-12 was seen (de la Maza and Carter, 1981). In all experiments, the inhibition seen with DI particles was as great as or greater than that seen with infectious AAV particles. Inhibition of oncogenicity was also seen when purified AAV DNA was used instead of complete virions. Furthermore, DNA from DI particles as well as sonicated viral DNA inhibited oncogenicity. No effect was seen when

poly(I:C), an interferon inducer, was used instead of AAV DNA, suggesting that interferon was not involved in the inhibition phenomenon. Since AAV DI particles, DI particle DNA, and sheared infectious viral DNA all inhibited adenovirus oncogenicity, it has been suggested that the termini of AAV DNA or sequences close to the termini are involved in the inhibition phenomenon (de la Maza and Carter, 1981; Cukor *et al.*, 1984).

As mentioned above, only certain serotypes of adenovirus are oncogenic *in vivo,* whereas all serotypes can transform rodent cells *in vitro.* The tumorigenicity of cells transformed by adenovirus *in vitro* is related to the type of virus used to transform the cells. Cells transformed with adenovirus-5, a nononcogenic strain, are less oncogenic than cells transformed with adenovirus-12, a highly oncogenic strain (Freeman *et al.*, 1967a–c; McAllister *et al.*, 1969; Gallimore and Paraskeva, 1980; Van den Elsen *et al.*, 1982). Therefore, it is interesting to consider the changes that take place at the cellular level during adenovirus transformation. Furthermore, it is interesting to consider what changes may be made by AAV at the cellular level which would decrease tumorigenicity.

The region of the adenovirus genome required for cell transformation has been mapped to the left-hand 11 map units (Graham *et al.*, 1974; Sambrook *et al.*, 1974). This region contains two transcriptional units called *E1a* and *E1b*. Their promoters map at 1.5 and 4.5 map units, respectively (Wilson *et al.*, 1979). Analysis of adenovirus-transformed cells showed that this region of the genome is integrated into cellular DNA (Sambrook *et al.*, 1974). This region is highly conserved with respect to nucleotide sequence and structural organization among various serotypes of adenovirus (Bos *et al.*, 1981; Van Ormondt and Hesper, 1983). The *E1a* region encodes proteins of approximately 11, 25, 49, and 33–55 kDa (Levinson and Levine, 1977; Jochemsen *et al.*, 1980, 1982; Ross *et al.*, 1980). The *E1b* region encodes two protein of 19 and 55–58K (Bos *et al.*, 1981).

Studies with host-range mutants of adenovirus show that *E1b* expression is required for transformation (Graham *et al.*, 1978; Lassam *et al.*, 1979). However, studies using DNA transfection of specific restriction fragments showed that the left-hand 4.5% of the adenovirus genome, containing the *E1a* region only, is capable of partially transforming cells (Schrier *et al.*, 1979; Houweling *et al.*, 1980). Furthermore, adenovirus host-range mutants in the *E1a* region also only partially transform cells (Graham *et al.*, 1978; Lassam *et al.*, 1979). These results could be explained by postulating a requirement of functions from both *E1a* and *E1b* for cell transformation.

Oncogenes can be classified in one of two categories (Houweling *et al.*, 1980; van der Eb *et al.*, 1979; Triesman *et al.*, 1981; Rassoulzadegan *et al.*, 1982; Land *et al.*, 1983; Ruley, 1983). One type of oncogene provides an establishment function that is required for cell immortalization. The second type of oncogene, the transforming function, is required for full expression of the oncogenic phenotype. The *E1a* region provides the establishment function. Transforming genes, such as polyoma virus middle-T antigen and the cellular oncogene isolated from T24 human bladder carcinoma cells (T24 ha-*ras* 1), are unable to transform primary baby rat kidney cells alone. When these genes were cotransfected with *E1a,* however, cell transformation took place (Ruley, 1983). The *E1b* region encodes the transforming functions (Shiroki *et al.*, 1979; Houweling *et al.*, 1980; Van den Elsen *et al.*, 1982). Cotransfection of *E1a* and *E1b* resulted in cell transformation.

Differences between oncogenic and nononcogenic serotypes of adenovirus have been noted at the cellular level. Some of these involve the ability of adenovirus-transformed cells to escape immune surveillance (Schrier *et al.*, 1983; Bernards *et al.*, 1983b). The ability of transformed cells to escape immune surveillance does not entirely explain the difference between oncogenic and nononcogenic strains of adenovirus, however. A difference in oncogenic potential between adenovirus-5 and adenovirus-12 can also be seen when cells transformed by these viruses are injected into nude mice. Studies with chimeras mapped the region involved to the region encoding the large tumor antigen of *E1b* (Bernards *et al.*, 1982, 1983a). This antigen is a 58 kDa protein in adenovirus-5 and a 55 kDa protein in adenovirus-12 (Bos *et al.*, 1981). The 58 kDa T-antigen of adenovirus-5 is associated with a 53 kDa cellular protein (p53) in adenovirus-transformed cells (Sarnow *et al.*, 1982a). This is the same cellular protein that is associated with the SV40 T antigen in SV40-transformed cells (Lane and Crawford, 1979; Linzer and Levine, 1979) as well as being present in a variety of other transformed cell lines (DeLeo *et al.*, 1979; Rotter *et al.*, 1980; Benchimol *et al.*, 1982; Thomas *et al.*, 1983).

Experiments have been reported that were aimed at determining the effect of AAV on adenovirus transformation *in vitro.* Casto and Goodheart (1972) reported that AAV1 could inhibit transformation of hamster cells by either the human adenovirus-12 or the simian adenoviruses SA7 and SV11. Quantitation of the dose dependency of the inhibition phenomenon showed that a higher multiplicity of AAV infection was required to inhibit transformation by a given percentage than was required to inhibit adenovirus replication by that same percentage. Whether this represents a difference in the mechanism by

which AAV inhibits the two adenovirus functions or a difference in the mechanism by which adenovirus carries out these two functions is unknown.

When AAV is infected into cells already transformed by adenovirus, the cells lose some of their oncogenic phenotype. When hamster cells transformed with adenovirus-12 were infected with AAV2 and subsequently injected into newborn hamsters, a decrease in the percentage of animals with tumors from 53 to 25% was noted (de la Maza and Carter, 1981). These experiments were also performed using a hamster cell line named H14b which is transformed by adenovirus-5, (Williams, 1973; Goldman *et al.*, 1974). This cell line is anomalous in that, although it is transformed by adenovirus-5, it is still oncogenic when injected into newborn hamsters. When H14b cells were infected with AAV prior to injection into 4- to 5-day-old hamsters, between 17 and 40% of the animals developed tumors as opposed to the 75–100% levels seen when uninfected H14b cells were injected (Ostrove *et al.*, 1981). Additionally, the mean latent period increased from 21 to 45 days and the tumor volumes decreased as much as 1000-fold. This inhibition of oncogenicity was not observed with a chemically transformed cell line, DMBA-2R (Lausch and Rapp, 1971). The inhibition of oncogenicity of H14B cells was not total in that if the dosage of cells given to the animals was increased, the inhibition was no longer seen (Ostrove *et al.*, 1981). In addition to the decrease in tumorigenicity, AAV-infected H14b cells also demonstrated an increased anchorage dependency in growth and a temporary decrease in saturation density.

No replication of AAV DNA was detected in infected cells, consistent with the data of de la Maza and Carter (1981) that AAV replication is not required for inhibition of oncogenicity. Transcription of AAV DNA was seen, however. This is to be expected since the adenovirus helper function for transcription is provided by early region 1 which is expressed in these cells (Williams, 1973; Goldman *et al.*, 1974). Furthermore, the presence of transcription suggests that a limited amount of DNA replication may have taken place in order to create a double-stranded template for transcription.

Southern blot analysis showed that no alteration in the integrated adenovirus DNA occurred with AAV infection, and Northern blot analysis showed that no change in adenovirus transcript accumulation occurred either. Analysis of the tumor antigens by immunoprecipitation with antisera raised in hamsters against H14b cells showed an 80% reduction in the amount of 58 kDa T antigen present when H14b cells were infected with AAV.

The association of the 58 kDa T antigen with cellular p53 has already been discussed. Additionally, *E1b* has been implicated both in the

shutoff of host cell protein synthesis (Babiss and Ginsberg, 1984) and in the production of adenovirus proteins (Rowe *et al.*, 1984). The decreased level of *E1b* proteins seen with AAV infection could have many effects on the metabolism of the adenovirus-transformed cell. It has yet to be determined which of the effects is altered by AAV. This determination may indicate the specific *E1b* functions required for transformation.

The phenomenon of oncogenic inhibition by AAV is not restricted to adenovirus-transformed cells. A line of hamster embryo fibroblasts transformed by Herpes simplex virus type 2 (HSV-2) named 333-8-9 (Duff and Rapp, 1971a,b) were infected with AAV1 and inoculated into weanling Syrian hamsters (Cukor *et al.*, 1975). The mean latent period before tumor development rose from approximately 30 days in control animals to approximately 50 days in animals that received AAV-treated cells. The mean survival time of the group receiving AAV-treated cells was 50% greater than the mean survival time of the group receiving untreated cells. Adeno-associated virus had no effect on the growth rate of 333-8-9 cells *in vitro* as measured by cell count and protein synthesis.

Adeno-associated virus gene expression in the absence of adenovirus can be detected in 333-8-9 cells at the level of capsid protein synthesis (Mayor and Drake 1974; Blacklow *et al.*, 1975, 1978). Indirect immunofluorescence of AAV-infected 333-8-9 cells showed production of VP1 and VP3 but not VP2. This capsid protein synthesis was seen with AAV1 and AAV3 infection but not with AAV4.

The ability of AAV to mediate herpesvirus-induced oncogenicity is interesting in light of the possible association of Herpes simplex viruses with cervical neoplasia. Epidemiologic evidence has been accumulating that implicates an infectious agent as the cause of cervical carcinoma (Kessler, 1977; zur Hausen *et al.*, 1981). Evidence has also been accumulating that implicates Herpes simplex virus as that infectious agent. The evidence includes (1) the presence of HSV-2 DNA in cervical carcinoma biopsy samples (Frenkel *et al.*, 1972), (2) the presence of HSV-2 RNA and protein in premalignant and malignant cervical tissue (McDougall *et al.*, 1980; Elgin *et al.*, 1981; Maitland *et al.*, 1981), and (3) the presence of higher HSV-2 antibody titers in cervical cancer patients than in appropriately matched controls (Aurelian *et al.*, 1970; Rawls *et al.*, 1980). Additionally, studies of HSV *in vitro* have shown the virus to be an inducer of cell transformation (Duff and Rapp, 1971a), chromosomal aberrations (Hampar and Ellison, 1963), and DNA repair (Lorentz *et al.*, 1977; Nishiyama and Rapp, 1981).

Evidence is also accummulating that suggests the involvement of human papilloma viruses (HPVs) in cervical cancer. Biopsy material

has been examined for the presence of HPV DNA sequences and several biopsies have tested positive for HPV-16 and HPV-18 sequences (Durst *et al.*, 1983; Gissmann *et al.*, 1984; Crum *et al.*, 1984; zur Hausen *et al.*, 1984). It has long been known in animal systems that papilloma lesions can convert to carcinomas when exposed to potent carcinogens or radiation (Rous and Friedwald, 1944; Jarrett *et al.*, 1980). Therefore, it has been suggested (zur Hausen, 1982) that human cervical cancer is the result of the conversion of papilloma virus-induced cervical dysplasias into malignant tumors. Furthermore, it has been suggested that Herpes simplex virus can serve as an agent inducing this conversion. The mutagenic nature of HSV-2 is consistent with this hypothesis.

It is tempting to consider the possibility that, as an inhibitor of Herpes simplex virus oncogenicity, AAV may be inhibiting the herpesvirus-induced conversion of a papilloma lesion to a malignant tumor. It should be noted that the most recently isolated serotype of adeno-associated virus, AAV-5, was isolated from a flat penile condyloma (Bantel-Schaal and zur Hausen, 1984).

Two types of experiments have been designed to test the involvement of AAV in inhibiting herpes oncogenicity. A line of Chinese hamster embryo cells transformed by SV40, CO631 (Lavi 1981), can be induced to amplify its integrated SV40 sequences by chemical carcinogens. Additionally, it has been shown that HSV-1 can also induce this gene amplification (Schlehofer *et al.*, 1983a). Coinfection of CO631 cells with HSV-1 and AAV5 inhibited the amplification of the SV40 sequences (Schlehofer *et al.*, 1983b). This inhibition was seen when AAV5 was added at any time from several days before to 3.5 hours after HSV-1 infection. Surprisingly, AAV5 also inhibited the induction of SV40 sequences by dimethyl benzanthracene (DMBA). It did not inhibit the induction by either 4-nitroquinoline-1-oxide or benzo[*a*]pyrene. Not only did AAV5 inhibit induction by DMBA, AAV5 replicated in these cells. This was surprising since no apparent helper virus functions were present. This helper virus-free replication has not been seen in any other cell line subsequently tested with AAV2 (E. Winocour, personal communication).

The second type of experiment involved the seroepidemiology of AAV infection. In three different studies (Sprecher-Goldberger *et al.*, 1970, 1971; Mayor *et al.*, 1976; Georg-Fries *et al.*, 1984) patients' sera were tested for the presence of antibody to AAV1, AAV2, AAV3, and AAV5. The presence of antibody to AAV was lower in cancer patients than in normal patients. When a specific group of cervical cancer patients was tested (Mayor *et al.*, 1976) only 14% possessed antibody,

in contrast to greater than 80% of the control groups. In the case of AAV5, when the geometric mean titers were compared, the control patients exhibited approximately 3-fold higher titers than the cervical cancer patients (Georg-Fries *et al.*, 1984).

Although these experiments are indirect and represent preliminary data, they yield enticing results. Clearly the role of AAV in inhibiting herpesvirus and adenovirus oncogenicity requires more detailed molecular analysis before firm conclusions can be drawn.

VII. AAV as a Eukaryotic Cloning Vector

The development of recombinant DNA technology has provided a variety of useful tools to study gene expression. Prokaryotic cloning vectors made from bacterial plasmids or bacteriophage have allowed biologists to isolate specific genes of interest and to prepare large quantities of the DNA for biochemical analyses, such as nucleotide sequencing. In order to understand the functional significance of these sequences, however, it is necessary to develop a system in which cloned genes can be expressed in their natural environment. If the genes are from a mammalian source, it would be interesting to study their expression in mammalian cells.

Animal viruses have provided a basis from which to design vectors. Representatives from almost every family of DNA animal viruses and representatives of one group of RNA animal viruses have been used as mammalian vectors. One of the most popular families of viruses for this purpose has been the papovavirus family. Vectors have been made from SV40 (Goff and Berg, 1976, 1979; Hamer and Leder, 1979; Hamer *et al.*, 1979; Mulligan *et al.*, 1979; Mulligan and Berg, 1980) and from bovine papilloma virus (Sarver *et al.*, 1981, 1982; Di Meo *et al.*, 1982; Law *et al.*, 1982). Other viruses that have been used as vectors include adenoviruses (Thummel *et al.*, 1981), herpesviruses (Kwong and Frenkel, 1984), poxviruses (Mackett *et al.*, 1982; Panicalli *et al.*, 1983), and retroviruses (Weeks *et al.*, 1982; Joyner and Bernstein, 1983).

The potential of retrovirus vectors is particularly interesting. Although they are RNA viruses, retroviruses replicate using a DNA intermediate (Gilboa *et al.*, 1979a,b). This DNA intermediate integrates into host cell DNA in a conserved way; that is, the junction between viral and cellular DNA is through the viral terminal repeats, with very little rearrangement of internal sequences (Dhar *et al.*, 1980; van Beveren *et al.*, 1980; Shimotohno *et al.*, 1980). This suggests

that retroviruses may be used as efficient vehicles for transducii foreign genes into a cell and inserting those genes into the host chr mosome (Weeks *et al.*, 1982; Joyner *et al.*, 1983). Recently it has bee shown that, by infecting preimplantation embryos with retroviral ve tors, genes can be inserted into the germ line of mice (van der Putten *al.*, 1985; Jahner *et al.*, 1985).

In addition to being used as a vehicle for transducing and expressin genes, retroviruses can be used to map and clone cellular DNA s quences. It has been shown that retroviruses can insert into and mut genize cellular genes and that this provides a tool for cloning out th mutagenized genes (Varmus *et al.*, 1981; Jenkins *et al.*, 1981; Jaenisc *et al.*, 1983; Kuff *et al.*, 1983; Wolf and Rotter, 1984; Frankel *et al* 1985). This provides a powerful tool for gene mapping in mammalia cells similar to those provided by bacteriophage mu (Denaire *et al.* 1977; Faelen *et al.*, 1977), yeast ty elements (Roeder and Fink, 1980) or *Drosophila* P elements (Searles *et al.*, 1982) in their respective sys tems. The mutagenized gene could be cloned easily if the origina retrovirus was carrying a gene that would allow for prokaryotic selec tion—either a replication origin (Cepko *et al.*, 1984) or a bacteria suppressor tRNA gene (Reik *et al.*, 1985).

In spite of their potential advantages, retroviruses have certain drawbacks. The retrovirus long terminal repeats (LTRs) contain powerful enhancer/promoter elements that would promote constitutive expression of the gene being transduced. This may not always be desirable. The LTRs may also turn on cellular genes downstream from the point of integration (Quintrell *et al.*, 1980). If the downstream gene happens to be a cellular oncogene, the result could be cell transformation (Hayward *et al.*, 1981; Payne *et al.*, 1981; Neel *et al.*, 1981). Additionally, it has been shown that intron sequences of genes inserted into retrovirus vectors may be lost during transduction (Shimotohno and Temin, 1982). Finally, the last drawback of retrovirus vectors is that the genes transduced may not be easily rescued and amplified for further cloning.

As mentioned above, AAV may integrate into host cell DNA in the absence of helper virus. This integration event resembles that of retroviruses in that the viral–cellular junction is always through the viral termini (Cheung *et al.*, 1980; Berns *et al.*, 1982). Secondly, although some mutations within the internal viral sequences may occur (Cheung *et al.*, 1980), enough intact sequences must remain for efficient virus production upon superinfection with helper adenovirus (Hoggan *et al.*, 1972; Handa *et al.*, 1977). This suggests that AAV can be used as a transducing vector in much the same way as retroviruses.

The cloning of AAV into bacterial plasmids and the discovery that this type of plasmid was infectious upon co-infection with adenovirus (Samulski *et al.*, 1982; Laughlin *et al.*, 1983) has enabled researchers to manipulate the AAV genome in a variety of ways to construct vectors.

As mentioned above, three promotors exist in the AAV genome at map units 5, 19, and 40 (Laughlin *et al.*, 1979; Green *et al.*, 1980; Green and Roeder, 1980a,b; Lusby and Berns, 1982). Tratchin *et al.* (1984b) have shown that the p19 and p40 promoters can be used to express the prokaryotic gene for chloramphenicol acetyltransferase (CAT) in transient expression assays. In the assays, both p19 and p40 were active in the absence of adenovirus, but p19 was also further induced by the E1a region of the adenovirus genome.

There have been two reports of stable gene transfers by AAV vectors (Hermonat and Muzycka, 1984; Tratchin *et al.*, 1985). Both sets of experiments involved transduction of the bacterial neomycin resistance gene which confers resistance to the antibiotic geneticin on tissue culture cells (Colbere-Garapin *et al.*, 1981). Hermonat and Muzyczka (1984) placed the neomycin resistance gene under the control of the SV40 promoter/enhancer. The gene and control element were substituted for the capsid-encoding region of the AAV genome. Virus stocks were made by cotransfection of this plasmid with a capsid gene-complementing plasmid that contained a large insert of bacteriophage λ DNA into adenovirus-infected cells. The λ insert increased the size of the complementing genome so that it could not be packaged. Wild-type AAV could still be detected in the virus preparation however (approximately 50% of the virions), presumably due to the high level of recombination that takes place between mutants in AAV-infected cells (Hermonat *et al.*, 1984) or a packaging bias for wild-type virus (P. L. Hermonat and N. Muzyczka, personal communication).

The resultant virus stock was used to infect recipient cells in the absence of adenovirus. The transduced cells were then selected for on the basis of resistance to geneticin. A variety of conditions were tested to optimize the transduction frequency and it was found that, at a multiplicity of infection of 1000 (relative infectious units per cell) and an incubation time prior to selection of 7 days, a transduction efficiency as high as 10% could be reached. As with wild-type AAV, approximately 30% of the cell clones tested contained rescuable vector sequences upon superinfection with adenovirus. All of the cell clones contained vector sequences integrated in host cell DNA when tested by Southern blots. Deletions within the *rep* region were also seen by Southern blot analysis. In light of the recent evidence that AAV *rep* genes can inhibit expression of genes under the control of SV40 early

promoters (M. A. Labow, L. H. Graf, and K. I. Berns, unpublished observations; B. J. Carter, personal communication), it is possible that *rep* gene mutations may have been selected for inadvertently in this procedure. It should be noted that the increased selection time also increased the number of vector copies present in the integrated tandem repeat (P. A. Collis, P. L. Hermonat, and N. Muzyczka, personal communication). This correlates well with the fraction of rescuable clones, suggesting that multiple copies of *rep* may be required for rescue and replication under these conditions.

Tratschin *et al.* (1985) placed the neomycin resistance gene under the control of the p40 promotor of AAV. The transduction frequency was similar to that observed by Hermonat and Muzyczka (1984). Southern blot analysis revealed the vector had integrated in both single and multiple tandem copy forms.

Both reports show the possibility of gene transduction by AAV vectors. Some of the drawbacks to retrovirus vectors do not exist with AAV. First, the AAV promotors are located internally and can be altered or eliminated if desired. This would allow the study of genes regulated by their own promoters. Second, AAV integration has never been shown to activate cellular oncogenes. Third, the host range of AAV is greater than those of retroviruses. Finally, AAV is easily rescuable. This phenomenon would be very useful in cloning dominant selectable genes from cDNA libraries.

The major drawback to AAV is its size. The AAV genome is 4.7 kb in size. The terminal 6% of the genome is required in cis for replication (Samulski *et al.*, 1983; Lefebvre *et al.*, 1984) and possibly for integration (Cheung *et al.*, 1980; Grossman *et al.*, 1984, 1985). The *rep* region is required in *trans* for replication (Hermonat *et al.*, 1984; Tratschin *et al.*, 1984a; Senapathy *et al.*, 1984). Sizes of AAV vectors greater than approximately 110–115% are not efficiently packaged (P. L. Hermonat and N. Muzyczka, personal communication). This leaves approximately 2.5 kb of sequence that can be substituted. It may be possible to gain another few kilobases if the rep region can be eliminated through complementation, but this has not yet been demonstrated.

Prokaryotic molecular biology has required the development of many different kinds of cloning vectors and there is no reason to assume that the same is not true of eukaryotic molecular biology. Many different kinds of eukaryotic cloning and expression vectors have already been designed. Evidence is not accumulating to suggest that AAV-based cloning vectors may play a unique role in the study of mammalian genetics and gene regulation.

VIII. Conclusions

Adeno-associated virus was initially viewed as an interesting system to study because of its unusual biology and because of its small size and the simplicity of the virion. The latter suggested the possibility that the simplicity of structure might be reflected in a mode of replication that was also relatively simple. As has become increasingly apparent and is reflected in this article, the truth has proved to be quite the opposite. Not only does the small genome code for the few structural proteins needed, but it also codes for at least two proteins in an ORF required for DNA replication. In addition, although AAV gene expression and DNA replication are dependent upon helper virus functions, a significant amount of self-regulation of these functions also occurs. All of these functions must be accommodated within a genome which is even smaller than that of the polyomaviruses. Hence, it is not surprising that the same sequences must participate in multiple functions, much as has been found to be the case with the polyomaviruses. This pleiotropy of many sequences has therefore rendered the genetic analysis of the virus actually more complex than may be the case for more complex genomes where such information and functions have more chance to be spaced along the genome. In the case of AAV it has frequently been difficult to be sure of just how many functions may have been affected by a single change in the DNA sequence. What has emerged is a fascinating picture of multiple layers of regulation which interdigitate quite closely. One of the suggestions emanating from this complex scheme is that rather than AAV replication being a unique process, it is more a demonstration of generality at the level of mechanism. In support of this notion are the facts that all aspects of AAV function require helper virus function and the fact noted in the introduction that AAV and its two helper viruses are structurally almost totally distinct and represent all the families of nuclear viruses with linear DNA genomes.

The extensive mutual interactions between AAV and its helper viruses raise an interesting question about the relationship between AAV and the autonomous parvoviruses. Although only extremely limited sequence homology exists between viruses in the two genera, the structural similarities are quite striking. (1) Virion diameter, shape, and density are indistinguishable. (2) The linear single-stranded DNA genomes differ in length by approximately 10%. (3) Although the autonomous parvoviruses were initially reported to encapsidate only the minus strand, whereas AAV encapsidates both plus and minus strands with equal frequencies in different virions, it is now clear that the plus

strand may also be encapsidated by some autonomous parvoviruses, sometimes as frequently as the minus strand. (4) The palindrome at the 3′ end of the virion strand of the autonomous viruses is very similar in length and potential secondary structure to the palindrome sequence in the AAV inverted terminal repeat, yet the 5′ palindrome on the right end of the autonomous parvovirus minus strand is not a terminal repeat and is unique from any AAV sequence. In the case of the human autonomous parvovirus, B19, however, inverted terminal repeats are seen (Shade *et al.*, 1986). (5) The palindromes at both ends of the AAV genome are inverted during DNA replication, but only the 5′ palindrome of the autonomous parvovirus genome is similarly inverted during replication. (6) The 5′ palindrome has protein covalently linked at the termini during replication and is blocked when extracted from the virion. No such terminal protein or block has been demonstrated for AAV DNA. (7) Virions of both genera contain three coat proteins which have overlapping amino acid sequences and the same relative molecular weights. (8) Both genomes generate three general species of transcripts, although only two promoters have been demonstrated on the autonomous parvovirus genome as opposed to the three on the AAV genome. However, the two autonomous parvovirus promoters correspond in position to two of the AAV promoters. Also, a third promoter analogous to the p19 promoter of AAV has been found in B19 (Shade *et al.*, 1986). (9) The ORF arrangements appear very similar on the two genomes. (10) One or two early proteins have also been identified in the case of the autonomous parvoviruses. (11) The left-side ORF of the autonomous parvovirus H-1 can also serve as a trans-activator of capsid gene transcription (Rhode, 1985). With all of this similarity and the autonomy in replication of the parvoviruses, it is truly amazing that AAV replication requires the large number of helper virus functions that have been demonstrated. It is still unclear at the mechanistic level as to how different the replication of the two genera may actually be, but the serious possibility does exist that the two are significantly different at the regulatory level. If they are not, what is the significance then of the wide spectrum of helper virus functions that are needed? This is certainly one of the major questions outstanding about the molecular biology of these viruses.

The biology of AAV has become increasingly of interest as the relationship between it and the host cell and helper viruses have become better understood. No disease has been associated with AAV infection. Thus, AAV may be nearly an ideal parasite since the general notion is to use, but not abuse, the host for virus propagation. This aspect is certainly well recognized in general, as is the concept that some microoganisms may actually be of benefit to the host. However, AAV is the

first animal virus for which this positive function has been suggested. Whether or not the presence of the virus does protect the host from either the acute pathogenic properties of either of the helper viruses or from only possible tumorigenic induction has still not been conclusively demonstrated, but the properties of AAV in this respect in cell culture and model systems *in vivo* are certainly suggestive.

Just how frequent the presence of AAV is in the population is difficult to estimate because it only has been isolated in the case of concurrent adenovirus infection. However, the high incidence of antibodies in the human population, the ability of AAV to establish latent infections from which it can be efficiently rescued by helper virus infection, and the high frequency of latent infection demonstrable in primary cultures of monkey cells make it seem likely that AAV is frequently present in the population, and thus further studies on possible protection for people are probably highly justified. Thus, an understanding of AAV latent infection would be helpful from an epidemiological point of view. Knowledge of the basic mechanisms is also desirable because the AAV genome has many of the physical and biological properties of mobile genetic elements, whose presence in animals is becoming increasingly apparent.

In summary, AAV represents a system which is an excellent model for the study of the regulation of replication in vertebrate cells and of the complex interactions not only between cells and viruses, but also interactions between different viruses which occur naturally.

ACKNOWLEDGMENTS

We thank M. Labow, S. Litherland, and P. Traktman, for critical reading of the manuscript. We also thank P. Burfeind, J. Mallette, and R. Higgins for help in preparation of the manuscript. Some of the research discussed was supported by a grant to K.I.B. from the National Institutes of Health, AI-22251. R.A.B. was partially funded by grant AI-07110-05 from the U.S. Public Health Service.

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NOTE ADDED IN PROOF. We have learned (E. Winocour, personal communication) that some human and hamster cells when treated so as to prolong S phase will support complete AAV replication in the absence of a helper virus.

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